

Metadata of the article that will be visualized in OnlineFirst

| | | | |
|----|--------------------------|---|---|
| 1 | Article Title | Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in European seabass (<i>Dicentrarchus labrax</i>) juveniles | |
| 2 | Article Sub- Title | | |
| 3 | Article Copyright - Year | Springer Nature B.V. 2020 (This will be the copyright line in the final PDF) | |
| 4 | Journal Name | Journal of Applied Phycology | |
| 5 | Corresponding Author | Family Name | Valente |
| 6 | | Particle | |
| 7 | | Given Name | Luisa M.P. |
| 8 | | Suffix | |
| 9 | | Organization | Terminal de Cruzeiros do Porto de Leixões |
| 10 | | Division | CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental |
| 11 | | Address | Av. General Norton de Matos, S/N, Matosinhos 4450-208, Portugal |
| 12 | | Organization | Universidade do Porto |
| 13 | | Division | ICBAS, Instituto de Ciências Biomédicas de Abel Salazar |
| 14 | | Address | Rua de Jorge Viterbo Ferreira, 228, Porto 4050-313, Portugal |
| 15 | | e-mail | lvalente@icbas.up.pt |
| 16 | Author | Family Name | Batista |
| 17 | | Particle | |
| 18 | | Given Name | Sónia |
| 19 | | Suffix | |
| 20 | | Organization | Terminal de Cruzeiros do Porto de Leixões |
| 21 | | Division | CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental |
| 22 | | Address | Av. General Norton de Matos, S/N, Matosinhos 4450-208, Portugal |
| 23 | | Organization | Universidade do Porto |
| 24 | | Division | ICBAS, Instituto de Ciências Biomédicas de Abel Salazar |
| 25 | | Address | Rua de Jorge Viterbo Ferreira, 228, Porto 4050-313, Portugal |
| 26 | | e-mail | |
| 27 | Author | Family Name | Pintado |

| | | | |
|----|--------|--------------|---|
| 28 | | Particle | |
| 29 | | Given Name | Manuela |
| 30 | | Suffix | |
| 31 | | Organization | Universidade Católica Portuguesa/Centro Regional do Porto |
| 32 | | Division | CBQF, Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia |
| 33 | | Address | Rua Arquitecto Lobão Vidal, Apartado 2511, Porto 4202-401, Portugal |
| 34 | | e-mail | |
| 35 | | Family Name | Marques |
| 36 | | Particle | |
| 37 | | Given Name | Alexandra |
| 38 | | Suffix | |
| 39 | Author | Organization | Terminal de Cruzeiros do Porto de Leixões |
| 40 | | Division | CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental |
| 41 | | Address | Av. General Norton de Matos, S/N, Matosinhos 4450-208, Portugal |
| 42 | | e-mail | |
| 43 | | Family Name | Abreu |
| 44 | | Particle | |
| 45 | | Given Name | Helena |
| 46 | | Suffix | |
| 47 | Author | Organization | ALGAplus, Produção e Comercialização de algas e seus derivados Lda |
| 48 | | Division | |
| 49 | | Address | PCI-Via do Conhecimento, Ílhavo 3830-352, Portugal |
| 50 | | e-mail | |
| 51 | | Family Name | Silva |
| 52 | | Particle | |
| 53 | | Given Name | Joana L. |
| 54 | | Suffix | |
| 55 | Author | Organization | Allmicroalgae, Natural Products S.A |
| 56 | | Division | |
| 57 | | Address | Apartado 9 EC Pataias, Pataias 2449-909, Portugal |
| 58 | | e-mail | |
| 59 | Author | Family Name | Jessen |
| 60 | | Particle | |

| | | | |
|----|--------------------|--|---|
| 61 | | Given Name | Flemming |
| 62 | | Suffix | |
| 63 | | Organization | Technical University of Denmark |
| 64 | | Division | National Food Institute |
| 65 | | Address | Søltofts Plads, Building 221, Kgs. Lyngby DK-2800, Denmark |
| 66 | | e-mail | |
| 67 | | Family Name | Tulli |
| 68 | | Particle | |
| 69 | | Given Name | Francesca |
| 70 | | Suffix | |
| 71 | Author | Organization | University of Udine |
| 72 | | Division | Department of Agriculture, Food, Environment and Animal Science |
| 73 | | Address | Udine, Italy |
| 74 | | e-mail | |
| 75 | | Received | 4 March 2020 |
| 76 | Schedule | Revised | 15 June 2020 |
| 77 | | Accepted | 15 June 2020 |
| 78 | Abstract | <p>Algae are natural sources of nutrients, but the presence of anti-nutritional factors often compromises nutrient apparent digestibility coefficients (ADCs) in several fish species. In this study, physical-mechanical and enzymatic technological processing was applied to two seaweeds (<i>Gracilaria gracilis</i> and <i>Ulva rigida</i>) and three microalgae (<i>Nannochloropsis oceanica</i>, <i>Chlorella vulgaris</i>, and <i>Tetraselmis</i> sp.) in order to evaluate its effectiveness in improving nutrient ADC values in diets for European seabass. A practical commercial-based diet was used as reference (REF) and experimental diets were prepared by replacing 30% of REF diet with each test alga used either intact or after processing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fast performance liquid chromatography (FPLC) analyses revealed that enzymatic processing was more effective than the physical one in changing the protein and peptides composition, increasing the amount of low-molecular-weight compounds in seaweeds and <i>N. oceanica</i> microalgae. Protein digestibility was significantly affected by algae species and in the case of the microalgae by the technological process. <i>Gracilaria gracilis</i> is better digested than <i>U. rigida</i> and physical processing enhanced protein and energy ADC values. <i>Nannochloropsis oceanica</i> and <i>C. vulgaris</i> are better digested than <i>Tetraselmis</i> sp.; the highest protein and energy ADCs were observed in diets containing enzymatically processed <i>N. oceanica</i> (NAN-ENZ) and physically processed <i>C. vulgaris</i> (CHLO-PHY), followed by the diet with physically processed <i>Tetraselmis</i> sp. (TETR-PHY). Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae by fish, by selecting the most adequate method to disrupt the cell wall. Moreover, the physical-mechanical and enzymatic technological processes used in this study are scalable to the industrial level.</p> | |
| 79 | Keywords separated | Algae - Antinutritional factors (ANFS) - Aquafeeds - Cell wall-rupture - | |

| | | |
|----|--------------------------|---|
| | by ' - ' | Nutrient digestibility (ADC) - Novel ingredients |
| 80 | Foot note information | Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. |

Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in European seabass (*Dicentrarchus labrax*) juveniles

Sónia Batista^{1,2} · Manuela Pintado³ · Alexandra Marques¹ · Helena Abreu⁴ · Joana L. Silva⁵ · Flemming Jessen⁶ · Francesca Tulli⁷ · Luisa M.P. Valente^{1,2}

Received: 4 March 2020 / Revised and accepted: 15 June 2020
© Springer Nature B.V. 2020

Abstract

Algae are natural sources of nutrients, but the presence of anti-nutritional factors often compromises nutrient apparent digestibility coefficients (ADCs) in several fish species. In this study, physical-mechanical and enzymatic technological processing was applied to two seaweeds (*Gracilaria gracilis* and *Ulva rigida*) and three microalgae (*Nannochloropsis oceanica*, *Chlorella vulgaris*, and *Tetraselmis* sp.) in order to evaluate its effectiveness in improving nutrient ADC values in diets for European seabass. A practical commercial-based diet was used as reference (REF) and experimental diets were prepared by replacing 30% of REF diet with each test alga used either intact or after processing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fast performance liquid chromatography (FPLC) analyses revealed that enzymatic processing was more effective than the physical one in changing the protein and peptides composition, increasing the amount of low-molecular-weight compounds in seaweeds and *N. oceanica* microalgae. Protein digestibility was significantly affected by algae species and in the case of the microalgae by the technological process. *Gracilaria gracilis* is better digested than *U. rigida* and physical processing enhanced protein and energy ADC values. *Nannochloropsis oceanica* and *C. vulgaris* are better digested than *Tetraselmis* sp.; the highest protein and energy ADCs were observed in diets containing enzymatically processed *N. oceanica* (NAN-ENZ) and physically processed *C. vulgaris* (CHLO-PHY), followed by the diet with physically processed *Tetraselmis* sp. (TETR-PHY). Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae by fish, by selecting the most adequate method to disrupt the cell wall. Moreover, the physical-mechanical and enzymatic technological processes used in this study are scalable to the industrial level.

Keywords Algae · Antinutritional factors (ANFS) · Aquafeeds · Cell wall-rupture · Nutrient digestibility (ADC) · Novel ingredients

Introduction

The sustainable growth of aquaculture largely depends on the use of novel nutrient sources to replace fish meal (FM) and

fish oil (FO), without compromising fish growth and welfare, and still assuring the nutritional value of end products (Naylor et al. 2009). Plants have been largely used to partially replace FM and FO, but lack omega-3 LC-PUFA (Turchini et al.

✉ Luisa M.P. Valente
lvalente@icbas.up.pt

¹ CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal

² ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

³ CBQF, Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Centro Regional do Porto, Rua Arquitecto Lobão Vidal, Apartado 2511, 4202-401 Porto, Portugal

⁴ ALGAplus, Produção e Comercialização de algas e seus derivados Lda, PCI-Via do Conhecimento, 3830-352 Ílhavo, Portugal

⁵ Allmicroalgae, Natural Products S.A, Apartado 9 EC Pataias, 2449-909 Pataias, Portugal

⁶ National Food Institute, Technical University of Denmark, Søtofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark

⁷ Department of Agriculture, Food, Environment and Animal Science, University of Udine, Udine, Italy

2010) and directly compete with animal and human nutrition (Gatlin et al. 2007). Algae-based ingredients have recently attracted the attention of the feed industry sector as sustainable sources of nutrients (Becker 2007; Wan et al. 2019) and they also contain many bioactive compounds like pigments, vitamins, and minerals with a large spectrum of biological activities (Holdt and Kraan 2011; Bellou et al. 2014; Araújo et al. 2016; Valente et al. 2016; Neto et al. 2018; Pereira et al. 2019; Batista et al. 2020). However, the ability of higher trophic level carnivorous fish, like European seabass *Dicentrarchus labrax*, to effectively extract nutrients from algal species is hampered by the high complexity of their cell walls, which may introduce anti-nutritional factors (Neto et al. 2018; Tibbetts 2018; Zheng et al. 2020); these may harm the intestinal tract and result in inflammation and reduced nutrient uptake (Araújo et al. 2016; Moutinho et al. 2018; Granby et al. 2020). Previous studies reported morphological alterations in the intestine of several fish species fed algal biomass, namely reduction of absorption area and epithelial degeneration (Atalah et al. 2007; Silva et al. 2015; Araújo et al. 2016; Moutinho et al. 2018). Moreover, several microalgae have highly recalcitrant cell walls and high carbohydrate content that negatively affect the activity of digestive enzymes (Skrede et al. 2011; Tibbetts 2018). Likewise, the presence of indigestible fibers in seaweeds (e.g., lectins), resistant to digestive enzymes, may affect their nutrient bioavailability (Wells et al. 2017; Zheng et al. 2020). The type of algal carbohydrates can affect the activity of digestive enzymes, in particular those located in the brush border membrane of the enterocyte, which is responsible for the final stages of degradation and assimilation of food (Perez-Jimenez et al. 2015).

To establish algal biomass as a sustainable next-generation ingredient, economically feasible processing technologies, able to disrupt cell walls, concentrate nutrients, and enhance nutrient bioavailability for fish, need to be developed (Tibbetts 2018). Application of such processing techniques would release proteins, lipids, and other naturally hydrophobic components and increase their digestion and nutrient absorption rate by fish (Tulli et al. 2017). Cell wall disruption and cell disintegration can be achieved through mechanical technologies (bead-beating, milling, ultrasonication, high-pressure homogenization, and spray-drying), thermal (microwave, autoclaving, and freezing), chemical (organic solvents, osmotic shock, and acid-alkali reactions), or biological processes (microbial degradation and enzymatic reactions) (Lee et al. 2012; Ometto et al. 2014; Günerken et al. 2015; Agboola et al. 2019). However, some of these disruption methods (e.g., bead milling, microwave, and ultrasonication) have high energy consumption, restricting their industrial applications (Günerken et al. 2015). Currently, enzymatic cell disruption has delivered effective and cost-competitive results when compared to mechanical and chemical cell

disruption methods (Demuez et al. 2015). The use of enzymes (e.g., lipase, pectinase, cellulase, and protease), single or mixed, and chemical procedures are considered of great interest as they can break down the polysaccharide and complex proteins allowing the release of smaller molecules such as peptides, some of them with bioactive properties, providing additional physiological health benefits to fish. The specificity of the selected enzymes plays an important role in the efficiency of cell degradation, leading to the frequent application of enzyme mixtures to target different structural molecules. However, the introduction of enzymes implies additional associated cost within the overall process (Demuez et al. 2015). For this reason, low-cost enzymatic mixtures used in combination with mechanical disruption may maximize the release of soluble protein and peptides in the algae biomass coupled with reduced processing price.

This work aims to evaluate the effectiveness of cost-effective physical-mechanical and enzymatic technological processes, applied to two seaweeds (*Gracilaria gracilis* and *Ulva rigida*) and three microalgae (*Nannochloropsis oceanica*, *Chlorella vulgaris*, and *Tetraselmis* sp.), in improving nutrient apparent digestibility coefficients (ADCs) in diets for European seabass juveniles.

Materials and methods

The present study was directed and performed by accredited scientists in laboratory animal science by the national competent authority (Direção Geral de Alimentação e Veterinária, DGAV) at a facility with permission to conduct experiments on fish, in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. All animal procedures were subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA-CIIMAR) and further approved by DGAV.

Ingredients

Two commercial IMTA-cultivated seaweed (*U. rigida* and *G. gracilis*) produced by ALGApplus (Ílhavo, Portugal) and three microalgae (*N. oceanica*, *C. vulgaris*, and *Tetraselmis* sp.) produced under industrial scale by Allmicroalgae (Pataias, Portugal) were dried by convection and by spray-dryer, respectively, before being used in this experiment. The selected algae were either used entirely (not processed, NO), or previously submitted to technological processing (Valente et al. 2019b) before being included in the test diets. The proximate composition, amino acid profile, and mineral content of each alga biomass are presented in Table 1. When

Table 1 Proximate composition, amino acid profile and mineral content of test algae without processing (% or kJ g⁻¹ dry matter basis)

| | <i>Ulva rigida</i> | <i>Gracilaria gracilis</i> | <i>Nannochloropsis oceanica</i> | <i>Chlorella vulgaris</i> | <i>Tetraselmis</i> sp. |
|--------------------------------|--------------------|----------------------------|---------------------------------|---------------------------|------------------------|
| Dry matter (% DM) | 83.3 | 89.7 | 93.8 | 96.1 | 95.2 |
| Crude protein | 15.1 | 34.5 | 34.7 | 54.0 | 26.3 |
| Crude fat | 1.2 | 0.6 | 10.2 | 9.9 | 1.4 |
| Ash | 30.1 | 19.4 | 36.1 | 12.7 | 34.1 |
| Carbohydrates ¹ | 36.9 | 35.2 | 12.8 | 19.5 | 33.4 |
| Gross energy | 11.5 | 15.4 | 16.2 | 20.9 | 13.3 |
| Neutral detergent fiber | 26.4 | 26.7 | 30.3 | 37.3 | 11.5 |
| EAA (% DM) | 7.2 | 13.9 | 20.1 | 27.3 | 12.6 |
| Arginine | 0.5 | 1.3 | 2.0 | 2.7 | 1.3 |
| Histidine | 0.1 | 0.2 | 0.6 | 0.4 | 0.3 |
| Lysine | 0.9 | 1.6 | 3.2 | 3.6 | 1.6 |
| Threonine | 0.8 | 1.7 | 1.9 | 3.3 | 1.2 |
| Isoleucine | 1.0 | 2.3 | 2.4 | 3.8 | 1.6 |
| Leucine | 1.1 | 1.9 | 3.1 | 4.1 | 2.1 |
| Valine | 1.7 | 3.1 | 5.1 | 6.1 | 2.8 |
| Methionine | 0.2 | 0.2 | vest | 0.6 | 0.3 |
| Phenylalanine | 0.8 | 1.7 | 1.8 | 2.7 | 1.4 |
| CEAA (% DM) | 0.8 | 1.4 | 1.7 | 2.3 | 1.1 |
| Cystine | vest | 0.4 | vest | 0.3 | 0.1 |
| Hydroxyproline | 0.2 | ND | vest | vest | vest |
| Proline | 0.6 | 1.0 | 1.7 | 2.0 | 1.0 |
| NEAA (% DM) | 7.0 | 10.9 | 12.9 | 22.2 | 7.6 |
| Alanine | 1.5 | 1.9 | 2.1 | 4.5 | 1.6 |
| Tyrosine | 0.6 | 1.3 | 1.1 | 2.7 | 0.7 |
| Aspartate | 1.4 | 2.6 | 2.9 | 4.5 | 1.5 |
| Glutamate | 1.9 | 2.4 | 3.9 | 5.7 | 1.6 |
| Glycine | 0.7 | 1.1 | 1.7 | 2.2 | 1.2 |
| Serine | 0.9 | 1.6 | 1.3 | 2.7 | 0.9 |
| Minerals (mg g ⁻¹) | | | | | |
| B | 0.03 | 0.08 | 0.05 | vest | 0.03 |
| Ca | 4.12 | 1.11 | 2.27 | 5.54 | 10.8 |
| Cu | 0.02 | 0.01 | 0.01 | 0.07 | 0.01 |
| Fe | 0.30 | 0.57 | 0.39 | 0.98 | 0.29 |
| K | 21.02 | 53.29 | 21.20 | 21.61 | 28.74 |
| Mg | 30.36 | 1.80 | 17.37 | 2.82 | 18.13 |
| Mn | 0.05 | 0.15 | 0.04 | 0.06 | 0.03 |
| Na | 24.07 | 8.51 | 55.97 | 7.07 | 44.02 |
| P | 1.58 | 4.64 | 7.27 | 21.12 | 5.61 |
| Zn | 0.01 | 0.02 | 0.07 | 0.19 | 0.03 |

EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; vest, vestigial amount of amino acid (< 0.01 mg g⁻¹)

¹ Calculated as 100 – (ash + crude protein + crude fat + moisture)

processed, the resulting product was entirely used as test ingredient. The peptide size distribution of aqueous extracts of the two seaweeds and three microalgae before and after processing is presented in Table 2.

Technological processing of algae

Two technological processes were applied to unprocessed algae biomass: a physical-mechanical rupture method (PHY) to

Table 2 Peptide size distribution and respective chromatogram area of aqueous extracts of the two seaweeds and three microalgae before and after processing

| | Algae | Processing | Chromatogram area according to size distribution | | | | Total area |
|-------|---------------------------------|------------|--|--------------|--------------|------------|------------|
| | | | > 12.3 kDa | 12.3–6.5 kDa | 6.5–0.19 kDa | < 0.19 kDa | |
| t2.4 | <i>Gracilaria gracilis</i> | NO | 6837 | 1814 | 14,183 | 47,784 | 70,618 |
| t2.5 | | PHY | 13,692 | 4676 | 19,002 | 58,561 | 95,930 |
| t2.6 | | ENZ | 15,935 | 9325 | 72,084 | 77,971 | 175,315 |
| t2.7 | <i>Ulva rigida</i> | NO | 6297 | 2950 | 5497 | 6095 | 20,839 |
| t2.8 | | PHY | 4084 | 4570 | 7581 | 9392 | 25,628 |
| t2.9 | | ENZ | 5200 | 9816 | 25,489 | 17,056 | 57,562 |
| t2.10 | <i>Nannochloropsis oceanica</i> | NO | 5401 | 2167 | 16,809 | 10,815 | 35,193 |
| t2.11 | | PHY | 5984 | 1575 | 16,157 | 10,541 | 34,257 |
| t2.12 | | ENZ | 22,080 | 5134 | 44,766 | 17,383 | 89,364 |
| t2.13 | <i>Chlorella vulgaris</i> | NO | 28,782 | 7120 | 34,655 | 54,550 | 125,106 |
| t2.14 | | PHY | 26,039 | 8079 | 47,256 | 84,715 | 166,089 |
| t2.15 | | ENZ | 20,944 | 7107 | 66,295 | 81,775 | 176,120 |
| t2.16 | <i>Tetraselmis</i> sp. | NO | 11,202 | 2637 | 8788 | 9344 | 31,971 |
| t2.17 | | PHY | 12,340 | 2773 | 8184 | 8486 | 31,783 |
| t2.18 | | ENZ | 13,089 | 4863 | 22,962 | 15,433 | 56,348 |

NO, not processed; PHY, physically processed; ENZ, enzymatically processed

efficiently disrupt cell walls using a vibratory grinding mill and enzymatic lysis using a cocktail of enzymes applied to the physically disrupted algae (ENZ). The PHY process relied on the use of a vibratory mill (Siebtechnik TS250, Geldern, Germany) with a solid dense puck and one ring, for 1–5 min, generating a disrupted algal suspension. In the ENZ process, physically disrupted algal biomass was hydrolyzed with a commercial low-cost enzymatic cocktail (containing lipase, pectinase, cellulase, and amylase, New Enzymes, Lda., Maia, Portugal) at a pH 6–7, for 3 h (Valente et al. 2019b). The yield in terms of recovered algae biomass was 79% and 99%, for the PHY and ENZ process, respectively. The recovered biomass was then dried using industrial methods already employed for each algal biomass: seaweeds were dehydrated in a pilot-scale tray dryer (Armfield UOP8, Ringwood, England), with an airflow of 0.6 m s⁻¹ maintained at 50 °C, until constant weight of the sample was achieved; microalgae were dried in a pilot-scale spray dryer (Niro Atomizer 2394, Copenhagen, Denmark) with a vanned wheel rotating at high speed and a concurrent drying chamber (0.8 m diameter and 0.6 m height). The dried algae biomass was collected in a single cyclone air separator system.

Experimental diets

Based on the known nutritional requirements of European seabass, a commercial-based diet was formulated and extruded by SPAROS Lda. (Olhão, Portugal) and used as basal

mixture (Table 3). To this mixture, 10 g kg⁻¹ chromic oxide (Cr₂O₃, Merck KGaA, Germany) was added as an inert marker for the evaluation of the apparent digestibility coefficient (ADC) of nutrients and energy. The reference diet (REF) consisted of 1000 g kg⁻¹ of the basal mixture (Table 4). Fifteen test diets were prepared by mixing 700 g kg⁻¹ of the basal mixture and 300 g kg⁻¹ of each test ingredient: *U. rigida* (DULV), *G. gracilis* (DGRA), *N. oceanica* (DNAN), *C. vulgaris* (DCHLO), and *Tetraselmis* sp. (DTRET); each test ingredient was either used unprocessed (NO) or after PHY or ENZ processes. The dried algal biomass (either not processed or processed) was ground (< 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany) prior addition to the basal mixture. Diets were manufactured with a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) to a pellet size of 3 mm and oil was added after the extrusion process. All batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) and stored at 4 °C until use. The formulation and proximate composition of the experimental diets are shown in Tables 4 and 5.

Digestibility trial

The digestibility trial was conducted at the Experimental Research Station of CCMAR (37° 00' N, 07° 58' W, Faro, Portugal) between November and December, with juvenile European seabass (*Dicentrarchus labrax*) obtained from

Table 3 Ingredient composition of the basal mixture

| Ingredients (%) | |
|--------------------------------------|------|
| Fishmeal 70 ¹ | 5.0 |
| Fishmeal 60 ² | 20.0 |
| Soy protein concentrate ³ | 12.0 |
| Pea protein concentrate ⁴ | 2.3 |
| Wheat gluten ⁵ | 5.5 |
| Corn gluten ⁶ | 8.0 |
| Soybean meal ⁷ | 15.0 |
| Rapeseed meal ⁸ | 5.0 |
| Wheat meal ⁹ | 11.3 |
| Fish oil ¹⁰ | 13.7 |
| Vit and min premix ¹¹ | 1.0 |
| Binder ¹² | 0.2 |
| Chromic oxide ¹³ | 1.0 |
| Dry matter (DM, %) | 95.2 |
| Crude protein (% DM) | 48.7 |
| Crude fat (% DM) | 13.5 |
| Carbohydrates (% DM) ¹⁴ | 22.8 |
| Gross energy (kJ g ⁻¹ DM) | 21.9 |
| Ash (% DM) | 10.2 |

¹ Peruvian fishmeal LT: 71.0% crude protein (CP), 11.0% crude fat (CF), EXALMAR, Peru;

² Fishmeal 60: 60% CP, 12% CF, Savinor SA, Portugal;

³ Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands;

⁴ Pea protein concentrate: Nutralys F85F, 78% CP, 1% CF, Roquette, France;

⁵ Wheat gluten: 84% CP, 1.3% CF, Roquette, France;

⁶ Corn gluten meal: 61.0% CP, 6.0% CF, COPAM, Portugal;

⁷ Soybean meal 48: Dehulled solvent extracted soybean meal: 47.7% CP, 2.2% CF, Cargill, Spain;

⁸ Rapeseed meal: 36% CP, 2.7% CF, PREMIX Lda, Portugal;

⁹ Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal;

¹⁰ Savinor S.A., Portugal;

¹¹ Vitamin and mineral premix: INVIVO 1%, Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg⁻¹ diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg⁻¹ diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings;

¹² Kielseguhr (natural zeolite): LIGRANA GmbH, Germany;

¹³ Cr₂O₃; Merck KGaA, Germany;

¹⁴ Calculated by estimation: 100 - (ash + crude protein + crude fat + moisture)

chromic oxide) and adapted over 4 weeks to the experimental conditions in quarantine. Subsequently, thirteen homogeneous groups of twelve fish (bodyweight 62 ± 8.6 g) were randomly distributed by thirteen tanks of 50 L with individual feces sedimentation columns (Guelph system), designed according to Cho and Slinger (1979) supplied with flow-through seawater. Fish were then adapted to the experimental conditions for 15 days (water temperature of 21 ± 1.8 °C, salinity of 35 g L⁻¹, flow rate at 3 L min⁻¹, and natural photoperiod corresponding to 10–11 h daylight length). After the adaptation period, fish were fed the experimental diets in a daily meal until visual satiation for 5 days a week during the feces collection period. Diets were tested in triplicate. All diets were accepted by the fish and no mortality was observed during the digestibility trial. About 30 min after feeding, every tank was carefully cleaned to assure that no uneaten pellet was left in the tanks and the sedimentation column. Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged (7200 rpm for 5 min) to eliminate water excess before freezing at -20 °C. Daily collection of the feces was performed for each experimental diet following previous seabass digestibility studies (Campos et al. 2018; Monteiro et al. 2018) until collecting the necessary amount of feces to perform all required analysis (8–17 days). Since the rearing system used consisted of thirteen tanks, this procedure was repeated over time until all ingredients were tested in triplicate. Each replicate was carried out in a different group of fish (tank) to reduce any tank effect. Fish were fasted for 24 h between the collecting period of different diets, allowing the first 5 days of feeding for adaption to the new diet. The remaining procedure was performed as described above. At the end of the trial, all feces were freeze-dried prior to analysis.

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979): $ADC (\%) = 100 \times (1 - (\text{dietary Cr}_2\text{O}_3 \text{ level} / \text{feces Cr}_2\text{O}_3 \text{ level}) \times (\text{feces nutrient or energy level} / \text{dietary nutrient or energy level}))$. ADC of dry matter was calculated as follows: $ADC (\%) = 100 \times (1 - (\text{dietary Cr}_2\text{O}_3 \text{ level} / \text{feces Cr}_2\text{O}_3 \text{ level}))$. The ADCs of nutrients and energy of the test ingredients were estimated according to NRC (2011): $ADC_{ing} (\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.7 \times D_{ref}) / (0.3 \times D_{ing}))]$; where ADC_{test} = ADC (%) of the experimental diet, ADC_{ref} = ADC (%) of the reference diet, D_{ref} = g kg⁻¹ nutrient (or kJ kg⁻¹ gross energy) of the reference diet (DM basis); D_{ing} = g kg⁻¹ nutrient (or kJ kg⁻¹ gross energy) of the test ingredient (DM basis). The digestible amino acids (DAAs) content of each algae meal was calculated as follows: $DAA (mg g^{-1} \text{ of DM}) = ADC \text{ of the amino acid in the test ingredient} \times AA_{ing}$, where AA_{ing} = mg g⁻¹ amino acid of the test ingredient (DM basis).

Acuicultura Y Nutricion De Galicia S.L. (Ortoño, Spain). Upon arrival, fish were fed the reference diet (without

Table 4 Formulation and proximate composition of the experimental diets

| t4.2 | | Experimental diets | | | | | | | | | | | | | | | |
|-------|--|--------------------|------|------|------|------|------|------|------|------|------|-------|------|------|-------|------|------|
| t4.3 | | REF | DULV | | | DGRA | | | DNAN | | | DCHLO | | | DTETR | | |
| t4.4 | | | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ |
| t4.5 | Basal mix (g kg ⁻¹) | 1000 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 | 700 |
| t4.6 | <i>Ulva rigida</i> | | 300 | | | | | | | | | | | | | | |
| t4.7 | <i>U. rigida</i> physically processed | | | 300 | | | | | | | | | | | | | |
| t4.8 | <i>U. rigida</i> enzymatically processed | | | | 300 | | | | | | | | | | | | |
| t4.9 | <i>Gracilaria gracilis</i> | | | | | 300 | | | | | | | | | | | |
| t4.10 | <i>G. gracilis</i> physically processed | | | | | | 300 | | | | | | | | | | |
| t4.11 | <i>G. gracilis</i> enzymatically processed | | | | | | | 300 | | | | | | | | | |
| t4.12 | <i>Nannochloropsis oceanica</i> | | | | | | | | 300 | | | | | | | | |
| t4.13 | <i>N. oceanica</i> physically processed | | | | | | | | | 300 | | | | | | | |
| t4.14 | <i>N. oceanica</i> enzymatically processed | | | | | | | | | | 300 | | | | | | |
| t4.15 | <i>Chlorella vulgaris</i> | | | | | | | | | | | 300 | | | | | |
| t4.16 | <i>C. vulgaris</i> physically processed | | | | | | | | | | | | 300 | | | | |
| t4.17 | <i>C. vulgaris</i> enzymatically processed | | | | | | | | | | | | | | 300 | | |
| t4.18 | <i>Tetraselmis</i> sp. | | | | | | | | | | | | | | | 300 | |
| t4.19 | <i>Tetraselmis</i> sp. physically processed | | | | | | | | | | | | | | | | 300 |
| t4.20 | <i>Tetraselmis</i> sp. enzymatically processed | | | | | | | | | | | | | | | | 300 |
| t4.21 | Proximate composition (% or kJ g ⁻¹ DM) | | | | | | | | | | | | | | | | |
| t4.22 | Dry matter (DM, %) | 95.2 | 89.8 | 92.3 | 92.3 | 91.7 | 92.8 | 91.6 | 90.0 | 93.8 | 93.0 | | 92.2 | 93.8 | 94.1 | 90.9 | 92.5 |
| t4.23 | Crude protein | 48.7 | 38.6 | 39.0 | 38.6 | 44.1 | 44.1 | 42.9 | 44.6 | 43.8 | 44.2 | | 51.2 | 50.1 | 48.9 | 41.7 | 41.2 |
| t4.24 | Crude fat | 13.5 | 11.9 | 10.3 | 9.3 | 11.0 | 10.5 | 11.6 | 12.3 | 11.9 | 11.7 | | 11.6 | 10.8 | 12.4 | 11.0 | 10.8 |
| t4.25 | Carbohydrates ¹ | 22.8 | 23.8 | 27.4 | 26.8 | 23.3 | 25 | 23 | 14.3 | 19.9 | 19.3 | | 18.3 | 21.8 | 21.9 | 20.2 | 24.2 |
| t4.26 | Gross energy | 21.9 | 19.0 | 20.4 | 21.1 | 20.3 | 23.2 | 22.5 | 22.0 | 21.0 | 22.9 | | 22.5 | 23.6 | 24.8 | 20.3 | 21.2 |
| t4.27 | Ash | 10.2 | 15.5 | 15.6 | 17.6 | 13.3 | 13.2 | 14.1 | 18.8 | 18.2 | 17.8 | | 11.1 | 11.1 | 10.9 | 18.0 | 17.6 |

REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30% *G. gracilis*; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. ¹ Calculated by estimation: 100 – (ash + crude protein + crude fat + moisture)

Chemical analysis

Each test ingredient, experimental diet, and feces were ground (feces were sifted) and homogenized before analysis. Proximate composition analysis was performed in duplicate. All samples were analyzed for dry matter (105 °C for 24 h), ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Germany; 500 °C for 5 h), crude protein (N × 6.25) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, USA), total lipid content according to Folch et al. (1957), and gross energy by an adiabatic bomb calorimeter (Werke C2000, IKA, Germany). Chromic oxide content in diets and feces was determined according to Bolin et al. (1952).

Algae crude fiber content was analyzed as neutral detergent fiber (NDF) according to ISO 16472:2006 (Robertson and Van Soest 1981; Van Soest and Robertson 1985); carbohydrates of the test ingredients were calculated by deducting the

sum of ash, CP, and total lipids from DM. The mineral content of the algae was determined according to USEPA (1995). Aliquots (0.3 g) of dry microalgae biomass were introduced in Teflon® microwave vessels and 9 mL of concentrated HNO₃ + 1.0 mL aqua regia was added. Samples were processed in a microwave digester (CEM Mars Xpress Matthews, USA) at 175 °C and elevated frequency of 2450 MHz. The temperature was kept at 170–180 °C for 10 min. After cooling, digested solutions were filtered through a PTFE filter (0.2 µm size), transferred into 20-mL volumetric flasks and stored at 5 °C for determination by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). A Varian Vista Pro axial instrument (Varian Inc., USA) equipped with a cross-flow nebulizer and auto-sampler was used. The calibration was performed using an ICP-standard 23 elements solution in 5% HNO₃ (Merck solution IV) using yttrium (Y) as an internal standard. The calibration curve and two blanks were run during each set of analyses, to check

Table 5 Amino acid profile of the experimental diets (% dry matter basis)

| t5.2 | | Experimental diets | | | | | | | | | | | | | | | |
|-------|----------------|--------------------|------|------|------|------|------|------|------|------|------|-------|------|------|-------|------|------|
| t5.3 | | REF | DULV | | | DGRA | | | DNAN | | | DCHLO | | | DTETR | | |
| t5.4 | | | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ |
| t5.5 | EAA (% DM) | 25.5 | 20.3 | 27.0 | 22.9 | 22.1 | 19.4 | 21.0 | 24.4 | 27.0 | 21.9 | 26.6 | 27.1 | 24.5 | 23.8 | 25.1 | 26.5 |
| t5.6 | Arginine | 2.9 | 2.6 | 3.4 | 2.9 | 3.0 | 2.5 | 2.2 | 2.3 | 3.2 | 2.4 | 3.4 | 3.1 | 2.9 | 3.0 | 2.8 | 3.4 |
| t5.7 | Histidine | 1.0 | 0.7 | 0.9 | 0.9 | 0.8 | 0.7 | 0.9 | 1.0 | 1.1 | 0.9 | 1.0 | 1.2 | 0.7 | 0.7 | 1.0 | 1.1 |
| t5.8 | Lysine | 3.9 | 2.3 | 3.8 | 3.2 | 2.7 | 2.2 | 2.4 | 2.5 | 3.0 | 2.5 | 3.4 | 2.7 | 3.0 | 3.6 | 2.7 | 3.1 |
| t5.9 | Threonine | 2.2 | 2.0 | 2.6 | 2.3 | 2.2 | 1.7 | 2.0 | 2.3 | 2.6 | 2.1 | 2.6 | 2.5 | 2.2 | 2.2 | 2.3 | 2.7 |
| t5.10 | Isoleucine | 3.6 | 2.3 | 3.6 | 2.4 | 2.6 | 2.5 | 2.7 | 3.3 | 3.9 | 3.1 | 2.9 | 3.4 | 3.2 | 3.3 | 3.5 | 3.3 |
| t5.11 | Leucine | 3.9 | 3.4 | 4.3 | 3.7 | 3.5 | 3.1 | 3.6 | 4.1 | 4.4 | 3.6 | 4.4 | 4.6 | 3.9 | 3.6 | 4.0 | 3.9 |
| t5.12 | Valine | 4.5 | 3.9 | 5.0 | 4.2 | 4.3 | 4.0 | 4.0 | 5.4 | 5.3 | 4.2 | 5.1 | 5.9 | 5.3 | 4.3 | 5.7 | 5.3 |
| t5.13 | Methionine | 1.1 | 1.2 | 0.9 | 1.2 | 1.1 | 0.9 | 1.2 | 1.3 | 1.1 | 0.9 | 1.4 | 1.2 | 1.1 | 1.0 | 1.1 | 1.3 |
| t5.14 | Phenylalanine | 2.3 | 1.8 | 2.3 | 2.0 | 1.8 | 1.7 | 1.9 | 2.2 | 2.4 | 2.1 | 2.4 | 2.5 | 2.1 | 2.1 | 2.0 | 2.4 |
| t5.15 | CEAA (% DM) | 3.8 | 2.9 | 3.5 | 3.5 | 3.0 | 2.8 | 3.1 | 3.6 | 3.8 | 3.3 | 3.6 | 3.8 | 3.4 | 3.2 | 3.5 | 3.5 |
| t5.16 | Cystine | 0.5 | 0.3 | vest | 0.4 | 0.4 | 0.3 | 0.3 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| t5.17 | Hydroxyproline | 0.6 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.5 | 0.3 | 0.3 |
| t5.18 | Proline | 2.7 | 2.3 | 3.2 | 2.7 | 2.4 | 2.2 | 2.5 | 3.0 | 3.1 | 2.6 | 3.0 | 3.1 | 2.7 | 2.3 | 2.9 | 2.9 |
| t5.19 | NEAA (% DM) | 20.0 | 17.9 | 22.7 | 20.3 | 20.1 | 18.0 | 19.9 | 20.3 | 23.0 | 18.9 | 22.0 | 25.2 | 20.0 | 21.7 | 23.0 | 23.5 |
| t5.20 | Alanine | 2.0 | 1.8 | 3.0 | 2.1 | 2.0 | 1.9 | 2.2 | 2.5 | 2.6 | 2.2 | 2.6 | 2.8 | 2.3 | 1.9 | 2.7 | 2.9 |
| t5.21 | Tyrosine | 1.7 | 1.2 | 1.5 | 1.5 | 1.3 | 1.1 | 1.3 | 1.5 | 1.6 | 1.4 | 1.7 | 1.8 | 1.5 | 1.7 | 1.4 | 1.7 |
| t5.22 | Aspartate | 4.2 | 3.2 | 4.3 | 3.6 | 4.1 | 3.5 | 3.6 | 4.3 | 4.7 | 3.8 | 4.7 | 4.8 | 4.0 | 4.4 | 4.7 | 4.2 |
| t5.23 | Glutamate | 7.4 | 7.7 | 8.6 | 8.8 | 8.6 | 8.0 | 9.0 | 7.4 | 9.2 | 7.1 | 8.1 | 10.8 | 8.0 | 9.7 | 9.4 | 10.0 |
| t5.24 | Glycine | 2.2 | 1.7 | 2.6 | 2.3 | 1.9 | 1.7 | 1.9 | 2.3 | 2.4 | 2.2 | 2.3 | 2.6 | 2.1 | 1.8 | 2.4 | 2.4 |
| t5.25 | Serine | 2.5 | 2.2 | 2.7 | 2.1 | 2.2 | 1.8 | 2.0 | 2.2 | 2.4 | 2.2 | 2.5 | 2.5 | 2.0 | 2.3 | 2.5 | 2.3 |

REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30% *G. gracilis*; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids. vest, vestigial amount of amino acid (< 0.1%)

the purity of the chemicals. The method detection limit (MDL) was calculated as $3 s/m$ (where s is the standard deviation of 10 replicate blanks and m is the slope of the calibration curve) for each element.

To measure the amino acid profile of test ingredients, experimental diets and feces samples were subjected to acid hydrolysis (6 M HCl) in an oven for 18 h at 110 °C. The hydrolysis was performed using an amount of the samples corresponding to 5–10 mg protein per mL HCl. After hydrolysis, the samples were cooled to room temperature (RT °C) and 100 µL was diluted with 1.5 mL 1 M NaCO₃ and filtered through a 0.2-µm syringe filter (Q-max PTFE, Ø13mm, Frisette ApS, Denmark) before derivatization using the EZ:Faast™ Amino Acid Analysis kit from Phenomenex (SA). The samples (50 µL) were then analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technology) according to the procedure described by Sabeena Farvin et al. (2010).

Protein pattern types of all algal extracts were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Mighty Small (Hoefer) slab cell according

to the method of (Laemmli 1970), using 12% acrylamide ($C = 2.6\%$, w/w) slab gels (1.5 mm thick). The algae extracts were obtained by adding 2 mL 1% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT) and 60 mM Tris HCl (pH 8.3) to 50 mg of each of the dried algae. After gentle shaking at room temperature for 1 h, samples were homogenized (Polytron PT 1200, Kinematica) for 30 s, boiled for 2 min, and incubated at room temperature for 30 min. The samples were then homogenized and boiled again for 2 min and centrifuged for 15 min at 20 °C at 20000×g. The supernatant was collected as sample extract. Extract aliquots were diluted 1:1 with sample buffer containing 125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA and bromophenol blue. Each lane was loaded with 20 µL sample, corresponding to 0.25 mg algae. Mark12 (Novex, USA) was used as molecular weight markers. The electrophoresis was run at 100 V for 15 min followed by 150 V for 1 h (max. 40 mA per gel) and afterwards the gels were stained using colloidal Coomassie Brilliant Blue, according to Rabilloud and Charmont (2000). To further evaluate the

effects of processing techniques on the algae, size exclusion chromatography by fast performance liquid chromatography (FPLC) was performed on algae aqueous extracts to characterize smaller proteins/peptides of molecular weights below approximately 20–30 kDa. The processed algae (100 mg) was extracted in 2 mL of water by homogenization (Polytron PT 1200, Kinematica) for 30 s, incubation at RT °C for 30 min followed by a new homogenization (30 s), and an incubation for 15 min (RT °C). The sample was then centrifuged for 15 min at 20 °C at 20000×*g* and the supernatant was filtered (0.2 µm) before analysis on fast performance liquid chromatography (FPLC) equipment (Äkta Purifier system with Frac 950 collector, GE Healthcare Life Sciences, UK). The sample (100 µL; corresponding to 5 mg algae) was injected onto a SuperdexTM peptide 10/300 GL column (GE Healthcare), using a 100 mM ammonium acetate, pH 8 as running buffer at a flow rate of 0.25 mL min⁻¹. Eluting compounds were detected at 215 nm. Cytochrome C (CytC, 12.3 kDa), aprotinin (6.5 kDa), and triglycine Gly3 (189 Da) were used as external molecular weights standards.

Statistical analysis

Seaweed and microalgae results are presented separately. ADCs data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA (for diets ADC) and two-way ANOVA (for ingredients ADC), with the statistical program IBM SPSS STATISTICS, 25.0 package, IBM Corporation, USA). When appropriate, individual means were compared using HSD

Tukey Test. When data did not meet the assumptions of ANOVA, a non-parametric test, Kruskal Wallis test was performed and the pairwise multiple comparison of mean ranks, were carried out to identify significant differences between groups. In all cases, the minimum level of significance was set at $p < 0.05$.

Results

The proximate composition of the test algae in their no-processed form (NO) varied enormously among species (Table 1). In terms of dry matter basis, crude protein content ranged from 15.1 (*U. rigida*) to 54.0% (*C. vulgaris*), crude fat varied between 0.6 (*G. gracilis*) and 10.2% (*N. oceanica*), and gross energy from 11.5 (*U. rigida*) to 20.9 kJ g⁻¹ (*C. vulgaris*). Neutral detergent fiber varied from 11.5 in *Tetraselmis* sp. to 37.3% in *C. vulgaris*. Ash content varied between 13 and 36%, being lowest in *C. vulgaris* followed by *G. gracilis*. The amino acid profile and mineral content also showed great variation among algae. *C. vulgaris* had the highest EAA content (27.3% DM), followed by *N. oceanica* (20.1%) and both were particularly rich in lysine (3.2–3.6) and valine (5.1–6.1). *U. rigida* had the lowest EAA content (7.2%), but is a rich source of Na, K, and Mg. Among selected algae, *G. gracilis* is the richest source of K and *C. vulgaris* is rich in P.

SDS PAGE was used to characterize the processing effects on the protein composition in the alga ingredients selected for this study by comparing the different algae to their processed counterparts (Fig. 1). The tested no-processed (NO) algae had different protein profiles (Fig. 1, lanes ULV-NO, GRA-NO,

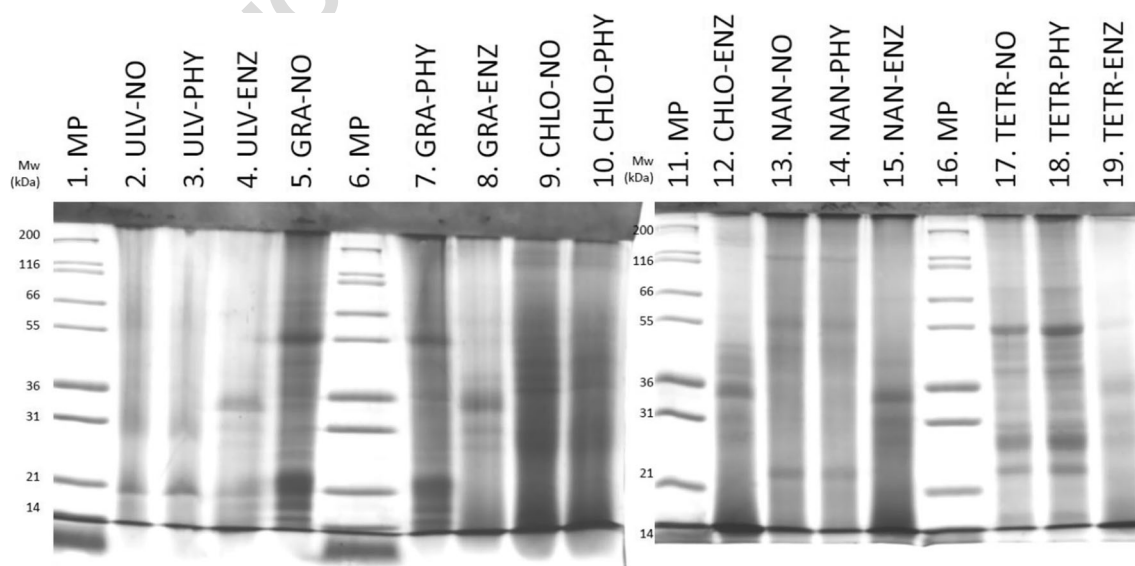


Fig. 1 SDS PAGE of the extracts of the fifteen algae ingredients included in this study ULV, *Ulva rigida*; GRA, *Gracilaria gracilis*; NAN, *Nannochloropsis oceanica*; CHLO, *Chlorella vulgaris*; TETR,

Tetraselmis sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; MP, Mark12TM was used as protein molecular weight marker

CHLO-NO, NAN-NO, and TETR-NO). The pattern of ULV-NO consisted of mainly two characteristic bands between 14 and 31 kDa, whereas the other four algae had more and better-defined bands distributed differently over most of the molecular weight range of the gels (14 to 200 kDa). The physical processing of the algae (Fig. 1: ULV-PHY, GRA-PHY, CHLO-PHY, NAN-PHY, and TETR-PHY) did not result in clear detectable changes in the protein profile of any of the five tested algae, compared with the no-processing groups. Contrarily, the enzymatic processing clearly changed the protein profile of all algae species resulting in a decrease in bands of high molecular weight proteins and an increase in low molecular weight proteins presented in the gels (Fig. 1, ULV-ENZ, GRA-ENZ, CHLO-ENZ, NAN-ENZ and TETR-ENZ) documenting an efficient effect of the enzyme treatment. FPLC analyses of all fifteen alga ingredients (Fig. 2) showed that the physical processing (PHY) had minor effects on the selected algae, compared to the enzymatic; low molecular compounds (peptide bond < 12.3 kDa based on integration of baseline subtracted FPLC profiles) evidenced

a 1.5-fold increase in *U. rigida* and *C. vulgaris* and 1.3-fold increase in *G. gracilis* (Table 2). However, the enzymatically process (ENZ) resulted in not only pronounced changes of the peaks' profiles of all the algae, but also in a generalized increase of low-molecular weight compounds (mainly peptides < 12.3 kDa) in all algae. For *U. rigida*, *G. gracilis*, and *N. oceanica*, this increase in peptides < 12.3 kDa was substantially higher (3.6, 2.5, and 2.3-fold increase, respectively; Table 2) than that perceived in either *C. vulgaris* (1.6-fold increase) or *Tetraselmis* sp. (2.1-fold increase).

The experimental diets, obtained by replacing 30% of the reference diet by each alga, had 39–51% protein, 9.3–12% fat, 19–25 kJ g⁻¹, 19.4–27% EAAs (Tables 4 and 5), reflecting the high variation observed in the nutritional value of each algae species.

The apparent digestibility coefficients (ADC) of macro nutrients, energy, and individual amino acids of the seaweed-rich diets fed to European seabass juveniles are reported in Table 6. The dry matter ADCs of the experimental diets varied between 38 and 67%, with diets containing *G. gracilis*

Fig. 2 Size exclusion chromatograms of aqueous extracts of the two seaweeds and three microalgae before and after processing. Eluting compounds were detected at 215 nm. Cytochrome c (CytC, 12.3 kDa), aprotinin (6.5 kDa) and triglycine (Gly3, 189 Da) were used as external standards for molecular weight. The largest molecule is eluted first from the column. mAU – milli absorbance units, higher mAU corresponds to larger amount of low molecular compounds absorbing at 215 nm (peptide bond). NO, not processed; PHY, physically processed; ENZ, enzymatically processed

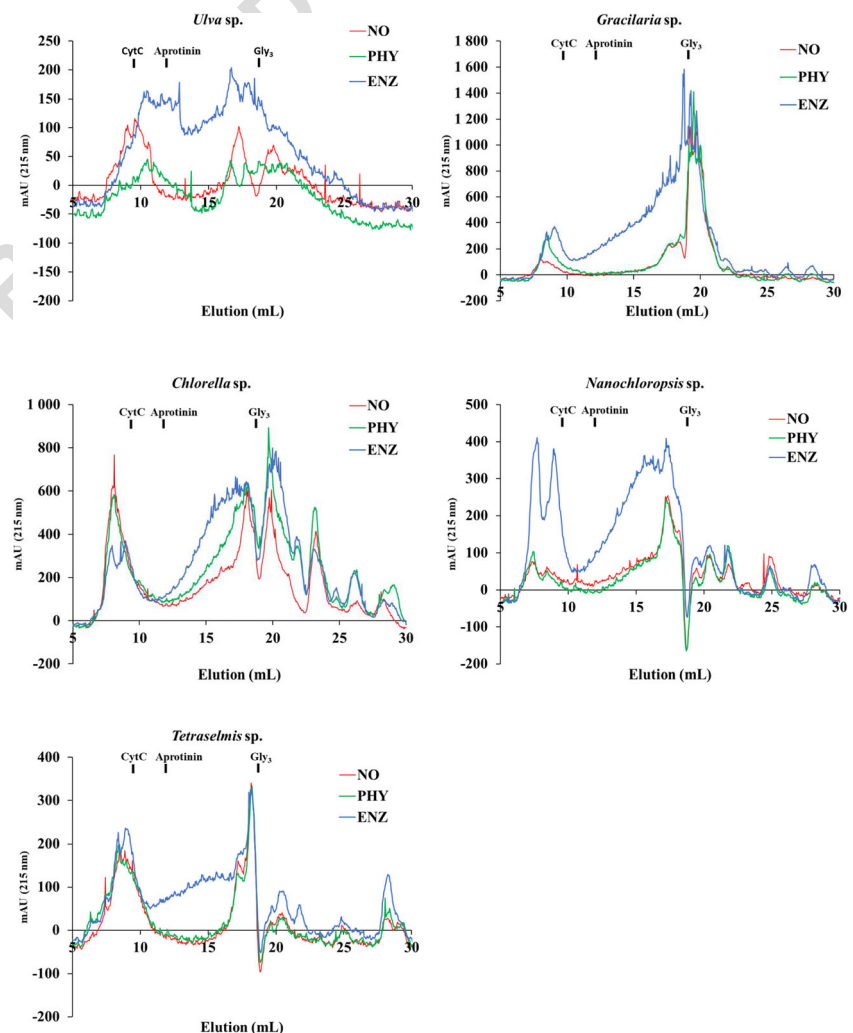


Table 6 Apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets containing seaweeds either used intact (NO) or after physic (PHY) or enzymatic (ENZ) processing

| t6.2 | | ADC (%) of experimental diets | | | | | | | | |
|-------|----------------|-------------------------------|---------------------|---------------------|--------------------|--------------------|--------------------|---------------------|-----|---------|
| t6.3 | | REF | DULV | | | DGRA | | | SEM | p value |
| t6.4 | | | NO | PHY | ENZ | NO | PHY | ENZ | | |
| t6.5 | Dry matter | 66.7 ^a | 49.0 ^{bc} | 38.2 ^c | 41.7 ^c | 60.0 ^{ab} | 62.2 ^{ab} | 60.2 ^{ab} | 2.5 | <0.001 |
| t6.6 | Protein | 94.5 ^a | 90.8 ^{ab} | 87.2 ^b | 87.4 ^b | 93.2 ^a | 93.9 ^a | 92.7 ^a | 0.7 | <0.001 |
| t6.7 | Lipids | 92.1 ^a | 88.8 ^a | 88.3 ^{ab} | 79.6 ^b | 94.3 ^a | 91.5 ^a | 91.9 ^a | 1.2 | 0.002 |
| t6.8 | Energy | 90.6 ^a | 87.0 ^{ab} | 85.8 ^b | 86.0 ^b | 86.1 ^{ab} | 89.7 ^{ab} | 88.6 ^{ab} | 0.5 | 0.01 |
| t6.9 | EAA | 95.6 ^a | 93.1 ^{ab} | 92.8 ^{ab} | 91.8 ^b | 93.7 ^{ab} | 94.5 ^{ab} | 93.6 ^{ab} | 0.3 | 0.02 |
| t6.10 | Arginine | 96.3 | 95.1 | 95.0 | 95.1 | 95.3 | 96.2 | 93.8 | 0.4 | 0.66 |
| t6.11 | Histidine | 92.0 | 88.6 | 87.4 | 84.2 | 92.3 | 90.0 | 89.4 | 1.1 | 0.48 |
| t6.12 | Lysine | 97.3 ^a | 94.1 ^{ab} | 94.5 ^{ab} | 95.1 ^{ab} | 93.5 ^{ab} | 93.6 ^{ab} | 93.0 ^b | 0.4 | 0.03 |
| t6.13 | Threonine | 95.2 | 92.9 | 92.9 | 92.6 | 93.6 | 93.9 | 93.9 | 0.3 | 0.05 |
| t6.14 | Isoleucine | 95.1 ^a | 91.3 ^{ab} | 92.8 ^a | 88.7 ^b | 92.3 ^{ab} | 94.7 ^a | 93.2 ^a | 0.5 | 0.002 |
| t6.15 | Leucine | 96.3 ^a | 94.3 ^{abc} | 92.9 ^{bc} | 91.7 ^c | 95.0 ^{ab} | 95.6 ^{ab} | 95.0 ^{abc} | 0.4 | 0.004 |
| t6.16 | Valine | 94.2 ^a | 90.9 ^{ab} | 90.8 ^{ab} | 89.3 ^b | 92.3 ^{ab} | 93.7 ^a | 92.5 ^{ab} | 0.4 | 0.003 |
| t6.17 | Methionine | 98.3 ^{ab} | 97.3 ^{bc} | 95.3 ^d | 96.4 ^{cd} | 98.9 ^a | 99.1 ^a | 98.9 ^a | 0.3 | <0.001 |
| t6.18 | Phenylalanine | 95.1 ^a | 92.3 ^{ab} | 92.0 ^c | 91.0 ^b | 91.6 ^b | 92.7 ^{ab} | 92.9 ^{ab} | 0.3 | 0.01 |
| t6.19 | CEAA | 97.0 ^a | 95.0 ^b | 94.2 ^b | 94.3 ^b | 95.1 ^b | 95.8 ^{ab} | 95.4 ^{ab} | 0.2 | 0.002 |
| t6.20 | Cystine | 98.3 ^a | 97.9 ^a | ND | 97.9 ^a | 93.8 ^b | 94.7 ^a | 94.0 ^a | 0.5 | <0.001 |
| t6.21 | Hydroxyproline | 96.9 ^a | 92.1 ^b | 89.7 ^b | 91.2 ^b | 96.5 ^a | 96.6 ^a | 96.4 ^a | 0.7 | <0.001 |
| t6.22 | Proline | 96.7 ^a | 94.9 ^{ab} | 94.9 ^{ab} | 94.1 ^b | 95.1 ^{ab} | 95.8 ^{ab} | 95.5 ^{ab} | 0.2 | 0.01 |
| t6.23 | NEAA | 95.3 ^a | 93.0 ^{ab} | 92.7 ^b | 92.9 ^b | 94.7 ^{ab} | 95.9 ^a | 95.2 ^{ab} | 0.3 | 0.01 |
| t6.24 | Alanine | 95.0 ^a | 91.6 ^{bc} | 92.9 ^{abc} | 90.7 ^c | 93.9 ^{ab} | 95.2 ^a | 94.8 ^a | 0.4 | 0.001 |
| t6.25 | Tyrosine | 96.8 ^{ab} | 94.5 ^{ab} | 94.3 ^b | 94.5 ^{ab} | 96.1 ^{ab} | 96.7 ^a | 95.8 ^{ab} | 0.3 | 0.02* |
| t6.26 | Aspartate | 94.9 ^a | 91.4 ^{ab} | 91.4 ^{ab} | 90.3 ^b | 93.9 ^{ab} | 94.6 ^a | 93.5 ^{ab} | 0.4 | 0.004 |
| t6.27 | Glutamate | 95.2 ^{ab} | 93.8 ^{ab} | 92.6 ^b | 94.1 ^{ab} | 95.5 ^{ab} | 97.0 ^a | 96.3 ^a | 0.4 | 0.01 |
| t6.28 | Glycine | 95.7 ^a | 92.8 ^{ab} | 93.9 ^b | 93.8 ^{ab} | 93.3 ^{ab} | 94.4 ^a | 94.4 ^{ab} | 0.3 | 0.17 |
| t6.29 | Serine | 95.6 ^a | 93.3 ^{ab} | 93.0 ^{ab} | 92.1 ^b | 94.0 ^{ab} | 95.0 ^{ab} | 94.2 ^{ab} | 0.3 | 0.02 |

Values are presented as mean \pm SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$). *without differences on post hoc test. ADC, apparent digestibility coefficient; REF, reference diet; DULV, diet with 30% *U. rigida*; DGRA, diet with 30% *G. gracilis*; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

(DGRA) not differing significantly from the REF diet, but those with *U. rigida* (DULV) displaying significantly lower values. Protein and energy digestibility values were not affected by the dietary inclusion of *G. gracilis*, but were significantly reduced when processed *U. rigida* was included in the diets (DULV-PHY and DULV-ENZ). The ADC of lipids was not strongly affected by the dietary inclusion of seaweeds, although DULV-ENZ (80%) had a significantly lower ADC value compared to the REF diet. The amino acid ADC values were generally high (> 90%) and followed the same trend reported for protein; diets including *G. gracilis* (DGRA) displayed similar values to the reference diet, but those including *U. rigida* showed decreased amino acid digestibility in particular the DULV-ENZ diet that have a significantly lower EAA ADC value compared to the REF diet.

The ADCs of the seaweeds are presented in Table 7. Overall, there was a significant effect of the tested seaweeds and technological process on nutrient digestibility, while the interaction of these factors was only significant in the case of lipid and methionine digestibility. *G. gracilis* was better digested by European seabass than *U. rigida*. Although the possessing technology had no significant impact on dry matter, protein, and energy ADC values, they increased by 19, 4, and 22%, in physically processed *G. gracilis* (GRA-PHY) in relation to the unprocessed algae. Contrarily, in *U. rigida*, the best ADC values were observed in unprocessed algae. The ADC of individual amino acids varied widely among algae and *G. gracilis* displayed the highest ADC values. The essential amino acids (EAA), conditionally essential amino acids (CEAA) and nonessential amino acids (NEAA) digestibility

Table 7 Apparent digestibility coefficients (ADC) of nutrients and energy of the tested seaweeds

| | | ULV | | | GRA | | | SEM | ANOVA | | |
|-------|----------------|---------------------|---------------------|----------------------|---------------------|---------------------|--------------------|------|---------|-------|-------|
| | | NO | PHY | ENZ | NO | PHY | ENZ | | S | P | S × P |
| t7.4 | Dry matter | 1.9 ^B | − 35.1 ^B | − 19.9 ^B | 43.4 ^A | 51.6 ^A | 44.2 ^A | 9.3 | < 0.001 | 0.52 | 0.24 |
| t7.5 | Protein | 62.7 ^B | 34.2 ^B | 27.2 ^B | 88.8 ^A | 92.0 ^A | 85.3 ^A | 6.9 | < 0.001 | 0.07 | 0.09 |
| t7.6 | Lipids | 0.5 ^a | 11.4 ^a | − 725.1 ^b | 216.3 ^a | 72.5 ^a | 82.7 ^a | 83.0 | 0.001 | 0.004 | 0.01 |
| t7.7 | Energy | 71.3 ^B | 64.7 ^B | 65.3 ^B | 71.2 ^A | 86.9 ^A | 81.5 ^A | 2.7 | 0.01 | 0.69 | 0.13 |
| t7.8 | EAA | 71.9 ^B | 69.7 ^B | 55.5 ^B | 85.4 ^A | 89.3 ^A | 80.1 ^A | 3.3 | 0.001 | 0.09 | 0.60 |
| t7.9 | Arginine | 78.3 | 80.8 | 78.6 | 89.4 | 95.6 | 75.3 | 3.8 | 0.37 | 0.54 | 0.64 |
| t7.10 | Histidine | 72.4 | ND | ND | 95.9 | 65.9 ^A | 61.3 | 17.1 | 0.38 | 0.56 | 0.67 |
| t7.11 | Lysine | 61.9 | 63.5 | 66.4 | 72.3 | 75.7 | 60.2 | 3.2 | 0.44 | 0.76 | 0.50 |
| t7.12 | Threonine | 78.5 ^B | 78.7 ^B | 75.4 ^B | 88.7 ^A | 88.8 ^A | 87.3 ^A | 1.8 | 0.004 | 0.77 | 0.96 |
| t7.13 | Isoleucine | 60.0 ^{Bxy} | 73.0 ^{Bx} | 28.1 ^{By} | 82.2 ^{Axy} | 93.0 ^{Ax} | 81.0 ^{Ay} | 5.8 | < 0.001 | 0.01 | 0.11 |
| t7.14 | Leucine | 77.4 ^{Bx} | 63.3 ^{Bxy} | 43.6 ^{By} | 89.1 ^{Ax} | 91.9 ^{Axy} | 85.1 ^{Ay} | 4.6 | < 0.001 | 0.02 | 0.07 |
| t7.15 | Valine | 70.9 ^B | 70.7 ^B | 55.2 ^B | 85.7 ^A | 91.7 ^A | 83.0 ^A | 3.4 | < 0.001 | 0.09 | 0.48 |
| t7.16 | Methionine | 85.3 ^b | 59.6 ^c | 65.8 ^c | 108.2 ^a | 115.4 ^a | 109.9 ^a | 5.4 | < 0.001 | 0.03 | 0.001 |
| t7.17 | Phenylalanine | 74.0 ^B | 71.6 ^B | 61.1 ^B | 80.5 ^A | 83.8 ^A | 81.9 ^A | 2.4 | 0.003 | 0.32 | 0.29 |
| t7.18 | CEAA | 72.2 ^B | 62.9 ^B | 60.2 ^B | 82.8 ^A | 86.4 ^A | 78.4 ^A | 2.9 | 0.001 | 0.26 | 0.42 |
| t7.19 | Cystine | 84.8 | ND | 71.4 | 78.8 | 78.9 | 71.0 | 3.9 | 0.76 | 0.56 | 0.79 |
| t7.20 | Hydroxyproline | 78.5 | 69.5 | 70.2 | ND | ND | ND | 2.0 | NA | 0.11 | NA |
| t7.21 | Proline | 74.8 ^B | 74.4 ^B | 61.2 ^B | 84.7 ^A | 89.1 ^A | 82.1 ^A | 2.8 | 0.003 | 0.14 | 0.55 |
| t7.22 | NEAA | 77.7 ^B | 74.3 ^B | 72.6 ^B | 91.8 ^A | 98.2 ^A | 94.3 ^A | 3.1 | < 0.001 | 0.86 | 0.62 |
| t7.23 | Alanine | 80.9 ^B | 86.4 ^B | 73.4 ^B | 91.3 ^A | 95.6 ^A | 94.1 ^A | 2.1 | < 0.001 | 0.07 | 0.13 |
| t7.24 | Tyrosine | 80.0 ^B | 79.5 ^B | 70.9 ^B | 94.0 ^A | 96.5 ^A | 90.0 ^A | 2.6 | < 0.001 | 0.17 | 0.81 |
| t7.25 | Aspartate | 66.4 ^B | 67.8 ^B | 57.4 ^B | 90.1 ^A | 93.3 ^A | 80.9 ^A | 3.8 | < 0.001 | 0.18 | 0.98 |
| t7.26 | Glutamate | 81.7 ^B | 62.5 ^B | 80.9 ^B | 97.9 ^A | 108.3 ^A | 111.8 ^A | 5.2 | 0.001 | 0.50 | 0.30 |
| t7.27 | Glycine | 70.2 | 79.7 | 76.9 | 81.9 | 87.4 | 85.1 | 2.4 | 0.07 | 0.44 | 0.93 |
| t7.28 | Serine | 78.8 ^B | 76.0 ^B | 66.9 ^B | 88.3 ^A | 92.5 ^A | 87.3 ^A | 2.6 | 0.001 | 0.27 | 0.51 |

Values are presented as mean ± SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$): differences among treatments (a, b); for a particular alga, differences caused by technological process (x, y); and for a particular technological process, differences caused by algae (A, B). ULV, *Ulva rigida*; GRA, *G. gracilis*; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. S, seaweed; P, process; EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

values of GRA-PHY were the highest, but without differing significantly from GRA ($P = 0.09$). The enzymatic process decreased the ability of seabass to digest leucine, irrespectively of the seaweed included in the diet, while methionine was significantly better digested in non-processed (ULV-NO) than in processed (ULV-PHY and ULV-ENZ) *Ulva* sp.

The ADCs of nutrients, energy, and amino acids of the experimental diets containing microalgae biomass are presented in Table 8. The dry matter ADCs of the experimental diets varied between 41 and 67%, with diets containing *N. oceanica* (DNAN) or *C. vulgaris* (DCHLO) not differing significantly from the REF diet. However, the dietary inclusion of *Tetraselmis* sp. biomass, either unprocessed (DTETR-NO) or enzymatically processed (DTETR-ENZ), resulted in a

significant decrease of dry matter ADC in relation to the REF diet (41–50% vs 67%, respectively). The dietary inclusion of unprocessed microalgae impaired protein ADC values, but after technological processing, diets DNAN-ENZ (93%), DCHLO-PHY (93%), and DCHLO-ENZ (92%) reached protein ADC values similar to those observed in the REF diet (95%). Energy ADC in DNAN-ENZ, DTETR-PHY, and in DCHLO diets did not differ from the REF diet. Lipid ADC values were reduced in DNAN and DTETR diets, irrespective of the processing method, but not in diets containing *C. vulgaris* (DCHLO). The amino acid ADC values were generally above 90%, except for histidine in DCHLO-ENZ, DTETR-NO, and DTETR-ENZ (> 83%). All CHLO diets had significantly lower lysine ADC value (90%) than the REF diet (97%). No differences were observed for total EAA and total

Table 8 Apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets containing microalgae either used intact (NO) or after physic (PHY) or enzymatic (ENZ) processing

| t8.2 | | ADC (%) of experimental diets | | | | | | | | | | | |
|-------|----------------|-------------------------------|---------------------|--------------------|---------------------|---------------------|--------------------|---------------------|--------------------|---------------------|---------------------|-----|---------|
| t8.3 | | REF | DNAN | | | DCHLO | | | DTETR | | | SEM | p value |
| t8.4 | | | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | | |
| t8.5 | Dry matter | 66.7 ^a | 56.4 ^{ab} | 62.9 ^{ab} | 64.5 ^{ab} | 59.0 ^{ab} | 65.7 ^a | 65.7 ^a | 40.9 ^c | 61.9 ^{ab} | 49.9 ^{bc} | 1.7 | <0.001 |
| t8.6 | Protein | 94.5 ^a | 91.5 ^{bc} | 91.2 ^{bc} | 93.0 ^{ab} | 91.6 ^{bc} | 92.6 ^{ab} | 92.3 ^{abc} | 89.8 ^c | 92.4 ^{bc} | 90.8 ^{bc} | 0.3 | <0.001 |
| t8.7 | Lipids | 92.1 ^a | 85.0 ^{bc} | 83.0 ^c | 85.0 ^{bc} | 90.4 ^a | 90.5 ^a | 89.5 ^{ab} | 84.4 ^c | 84.1 ^c | 84.8 ^c | 0.6 | <0.001 |
| t8.8 | Energy | 90.6 ^a | 87.2 ^{bc} | 87.2 ^{bc} | 89.7 ^{ab} | 88.0 ^{abc} | 90.5 ^a | 90.6 ^a | 82.0 ^d | 88.7 ^{abc} | 86.0 ^c | 0.5 | <0.001 |
| t8.9 | EAA | 95.6 | 93.9 | 94.0 | 93.7 | 92.9 | 93.8 | 93.1 | 91.9 | 94.2 | 93.5 | 0.2 | 0.11 |
| t8.10 | Arginine | 96.3 | 94.0 | 95.9 | 95.1 | 95.5 | 95.0 | 95.1 | 93.9 | 95.4 | 96.1 | 0.3 | 0.48 |
| t8.11 | Histidine | 92.0 | 92.0 | 90.3 | 89.7 | 89.3 | 91.3 | 83.3 | 83.3 | 90.4 | 87.2 | 1.1 | 0.56 |
| t8.12 | Lysine | 97.3 ^a | 94.8 ^{abc} | 95.5 ^{ab} | 94.9 ^{ab} | 90.7 ^{bc} | 89.0 ^c | 90.1 ^{bc} | 95.3 ^{ab} | 94.0 ^{abc} | 94.6 ^{abc} | 0.6 | 0.001 |
| t8.13 | Threonine | 95.2 | 92.8 | 93.9 | 93.5 | 93.3 | 94.2 | 93.3 | 91.4 | 93.7 | 93.8 | 0.2 | 0.05 |
| t8.14 | Isoleucine | 95.1 ^a | 94.1 ^{ab} | 93.6 ^{ab} | 93.3 ^{ab} | 91.4 ^b | 94.0 ^{ab} | 93.7 ^{ab} | 92.1 ^{ab} | 94.9 ^a | 92.6 ^{ab} | 0.3 | 0.01 |
| t8.15 | Leucine | 96.3 ^a | 94.3 ^{abc} | 94.2 ^{bc} | 94.0 ^{abc} | 94.0 ^{abc} | 95.1 ^{ab} | 94.3 ^{abc} | 91.5 ^c | 94.3 ^{abc} | 93.2 ^{bc} | 0.3 | 0.002 |
| t8.16 | Valine | 94.2 ^a | 93.2 ^a | 92.5 ^a | 92.2 ^a | 91.4 ^{ab} | 93.6 ^a | 92.9 ^a | 89.0 ^b | 94.0 ^a | 92.4 ^a | 0.3 | 0.001 |
| t8.17 | Methionine | 98.3 | 97.5 | 97.5 | 97.5 | 98.1 | 97.8 | 97.1 | 95.4 | 97.4 | 97.2 | 0.2 | 0.08 |
| t8.18 | Phenylalanine | 95.1 ^a | 93.1 ^a | 93.4 ^a | 93.9 ^a | 93.0 ^{ab} | 94.2 ^a | 93.2 ^a | 90.8 ^b | 93.1 ^a | 93.0 ^a | 0.2 | <0.001 |
| t8.19 | CEAA | 97.0 ^a | 96.3 ^a | 96.2 ^a | 96.1 ^a | 95.9 ^a | 96.5 ^a | 96.1 ^a | 94.0 ^b | 96.1 ^a | 95.3 ^{ab} | 0.2 | 0.001 |
| t8.20 | Cystine | 98.3 ^a | 96.3 ^c | 97.2 ^{bc} | 97.4 ^{ab} | 96.8 ^{bc} | 97.0 ^{bc} | 97.5 ^{ab} | 96.3 ^c | 97.4 ^{ab} | 97.4 ^{ab} | 0.1 | <0.001 |
| t8.21 | Hydroxyproline | 96.9 ^a | 96.0 ^{ab} | 94.3 ^{bc} | 95.2 ^{ab} | 95.8 ^{ab} | 95.1 ^{ab} | 95.8 ^{ab} | 95.4 ^{ab} | 95.4 ^{ab} | 92.7 ^c | 0.2 | <0.001 |
| t8.22 | Proline | 96.7 ^a | 96.4 ^a | 96.2 ^a | 96.0 ^a | 95.8 ^a | 96.5 ^a | 96.0 ^a | 93.3 ^b | 96.0 ^a | 95.3 ^{ab} | 0.2 | 0.001 |
| t8.23 | NEAA | 95.3 | 94.3 | 95.0 | 94.6 | 93.8 | 95.3 | 94.1 | 93.5 | 95.0 | 94.5 | 0.2 | 0.27 |
| t8.24 | Alanine | 95.0 ^a | 94.2 ^a | 94.0 ^a | 94.3 ^a | 93.3 ^a | 94.7 ^a | 93.5 ^a | 89.8 ^b | 94.7 ^a | 94.2 ^a | 0.3 | 0.001 |
| t8.25 | Tyrosine | 96.8 ^a | 95.2 ^{ab} | 95.4 ^{ab} | 95.5 ^{ab} | 94.4 ^b | 95.9 ^{ab} | 95.2 ^{ab} | 94.4 ^b | 95.2 ^{ab} | 95.6 ^{ab} | 0.2 | 0.01 |
| t8.26 | Aspartate | 94.9 | 93.6 | 94.1 | 93.6 | 93.2 | 94.3 | 93.0 | 92.6 | 94.5 | 92.7 | 0.2 | 0.19 |
| t8.27 | Glutamate | 95.2 | 94.8 | 96.0 | 94.8 | 94.3 | 96.1 | 94.8 | 95.4 | 95.4 | 95.4 | 0.2 | 0.69 |
| t8.28 | Glycine | 95.7 ^a | 94.3 ^a | 94.3 ^a | 95.5 ^a | 93.3 ^a | 94.9 ^a | 94.6 ^a | 90.4 ^b | 95.2 ^a | 94.4 ^a | 0.3 | <0.001 |
| t8.29 | Serine | 95.6 ^a | 93.2 ^{ab} | 94.2 ^{ab} | 94.3 ^a | 93.6 ^{ab} | 94.5 ^{ab} | 93.3 ^{ab} | 91.9 ^b | 94.9 ^a | 93.8 ^{ab} | 0.2 | 0.02 |

Values are presented as mean \pm SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$). ADC, apparent digestibility coefficient; REF, reference; DNAN, diet with 30% *N. oceanica*; DCHLO, diet with 30% *C. vulgaris*; DTETR, diet with 30% *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

NEAA ADCs, between the different dietary treatments. However, CEAA ADC in diet DTETR-NO was significantly lower (94%) than the REF diet (97%).

When considering the digestibility of microalgae (Table 9), overall, a significant effect of the tested seaweeds, technological process, and interaction of both factors on nutrient digestibility was observed. *Chlorella vulgaris* and *N. oceanica* generally had higher nutrient ADC values compared to *Tetraselmis* sp. Unprocessed microalgae had the lowest nutrient and energy digestibility values. Technological processing irrespective of the method applied, significantly ($p < 0.05$) improved microalgae dry matter digestibility (> 50% increase). The highest protein ADC values were registered in NAN-ENZ, CHLO-PHY, and CHLO-ENZ (> 88%), showing an

increase of 8, 4, and 3%, respectively, in relation to their unprocessed counterparts. The highest increase in protein ADC was observed in *Tetraselmis* sp. after physical processing (TETR-PHY, 20% increase). Technological processing dramatically enhanced energy ADC values in relation to unprocessed algae: 14% increase in NAN-ENZ; 11% in both CHLO-PHY and CHLO-ENZ; 66% in TETR-PHY and 40% in TETR-ENZ. The highest energy ADC values were observed in CHLO-PHY and CHLO-ENZ (> 90%). *Tetraselmis* sp. had the lowest energy (49%) ADC's, which was significantly enhanced ($p < 0.05$) after the physical process (66% increase, in relation to the unprocessed microalgae). Lipid ADC values of *Tetraselmis* sp. were significantly lower than the other microalgae and were extremely

Table 9 Apparent digestibility coefficients (ADC) of nutrients and energy of the tested microalgae

| | | NAN | | | CHLO | | | TETR | | | SEM | ANOVA | | |
|-------|----------------|---------------------|---------------------|----------------------|---------------------|--------------------|---------------------|----------------------|----------------------|----------------------|------|---------|---------|---------|
| | | NO | PHY | ENZ | NO | PHY | ENZ | NO | PHY | ENZ | | M | P | M × P |
| t9.4 | Dry matter | 32.0 ^{Ay} | 53.6 ^{Ax} | 59.4 ^{Ax} | 41.2 ^{Ay} | 63.4 ^{Ax} | 63.4 ^{Ax} | − 19.1 ^{By} | 51.4 ^{Bx} | 12.5 ^{Bx} | 5.8 | < 0.001 | 0.001 | 0.09 |
| t9.5 | Protein | 81.6 ^{ab} | 81.0 ^{abc} | 87.9 ^a | 85.5 ^{ab} | 88.6 ^a | 87.6 ^a | 69.7 ^c | 83.6 ^{ab} | 73.7 ^{bc} | 1.4 | < 0.001 | 0.03 | 0.02 |
| t9.6 | Lipids | 63.1 ^a | 56.1 ^a | 63.8 ^a | 84.9 ^a | 81.2 ^a | 78.4 ^a | − 92.4 ^b | − 101.2 ^b | − 795.0 ^c | 52.9 | < 0.001 | < 0.001 | < 0.001 |
| t9.7 | Energy | 76.2 ^{bc} | 76.6 ^{abc} | 87.0 ^{ab} | 81.5 ^{abc} | 90.4 ^a | 90.6 ^a | 48.9 ^d | 81.1 ^{abc} | 68.3 ^c | 2.5 | < 0.001 | < 0.001 | < 0.001 |
| t9.8 | EAA | 88.7 ^A | 89.2 ^A | 87.2 ^A | 86.9 ^{AB} | 90.1 ^{AB} | 87.2 ^{AB} | 74.3 ^B | 88.1 ^B | 82.7 ^B | 1.2 | 0.03 | 0.10 | 0.27 |
| t9.9 | Arginine | 86.1 | 94.6 | 90.6 | 93.2 | 92.2 | 92.8 | 81.4 | 90.9 | 95.0 | 1.3 | 0.49 | 0.13 | 0.33 |
| t9.10 | Histidine | 92.0 | 83.6 | 78.8 | 74.4 | 88.2 | 50.3 | 59.4 | 78.7 | 49.3 | 6.6 | 0.46 | 0.38 | 0.93 |
| t9.11 | Lysine | 87.5 ^A | 90.5 ^A | 86.4 ^A | 73.8 ^B | 71.7 ^B | 73.8 ^B | 84.2 ^{AB} | 76.2 ^{AB} | 76.8 ^{AB} | 1.9 | 0.01 | 0.77 | 0.83 |
| t9.12 | Threonine | 86.2 ^{ABy} | 90.5 ^{ABx} | 88.7 ^{ABxy} | 90.3 ^{Ay} | 92.3 ^{Ax} | 89.0 ^{Axy} | 74.8 ^{By} | 87.8 ^{Bx} | 87.5 ^{Bxy} | 1.2 | 0.01 | 0.02 | 0.10 |
| t9.13 | Isoleucine | 90.5 ^{abc} | 88.4 ^{abc} | 86.7 ^{abc} | 83.4 ^{abc} | 91.9 ^{ab} | 89.7 ^{abc} | 76.5 ^c | 93.9 ^a | 78.0 ^{bc} | 1.4 | 0.04 | 0.01 | 0.02 |
| t9.14 | Leucine | 88.5 ^a | 88.1 ^{ab} | 86.0 ^{ab} | 89.1 ^a | 92.6 ^a | 89.2 ^a | 71.1 ^c | 85.6 ^{ab} | 77.4 ^{bc} | 1.4 | < 0.001 | 0.01 | 0.04 |
| t9.15 | Valine | 91.1 ^a | 88.6 ^a | 87.5 ^a | 86.7 ^a | 92.7 ^a | 90.5 ^a | 69.1 ^b | 93.3 ^a | 86.1 ^a | 1.5 | 0.004 | 0.001 | < 0.001 |
| t9.16 | Methionine | 61.4 | ND | 91.2 | 97.1 | 95.8 | 67.3 | 73.9 | 89.9 | 86.5 | 3.7 | 0.77 | 0.24 | 0.04* |
| t9.17 | Phenylalanine | 87.4 ^{Ay} | 88.4 ^{Ax} | 89.9 ^{Axy} | 88.7 ^{Ay} | 92.3 ^{Ax} | 88.3 ^{Axy} | 74.4 ^{By} | 85.0 ^{Bx} | 83.7 ^{Bxy} | 1.1 | < 0.001 | 0.01 | 0.06 |
| t9.18 | CEAA | 93.1 ^a | 92.0 ^a | 91.3 ^a | 91.9 ^a | 94.5 ^a | 92.2 ^a | 68.9 ^b | 89.8 ^a | 81.7 ^{ab} | 1.7 | < 0.001 | 0.01 | 0.01 |
| t9.19 | Cystine | ND | ND | ND | 90.5 ^a | 91.8 ^a | 87.3 ^a | 42.5 ^b | 77.8 ^a | 80.7 ^a | 4.2 | < 0.001 | < 0.001 | < 0.001 |
| t9.20 | Hydroxyproline | ND | ND | ND | 35.6 | ND | ND | ND | 46.3 | ND | 7.4 | | 0.53** | |
| t9.21 | Proline | 95.1 ^a | 94.1 ^a | 93.1 ^a | 93.0 ^a | 95.9 ^a | 93.4 ^a | 71.8 ^b | 92.2 ^a | 86.1 ^a | 1.6 | < 0.001 | 0.01 | 0.01 |
| t9.22 | NEAA | 90.4 ^{Ay} | 93.6 ^{Ax} | 91.3 ^{Axy} | 90.5 ^{Ay} | 95.2 ^{Ax} | 90.6 ^{Axy} | 82.1 ^{By} | 93.6 ^{Bx} | 89.6 ^{Bxy} | 1.0 | 0.24 | 0.04 | 0.55 |
| t9.23 | Alanine | 92.4 ^a | 91.8 ^a | 92.7 ^a | 91.5 ^a | 94.4 ^a | 91.0 ^a | 74.3 ^b | 93.7 ^a | 91.7 ^a | 1.3 | 0.01 | 0.003 | 0.001 |
| t9.24 | Tyrosine | 89.3 ^A | 90.0 ^A | 90.0 ^A | 91.0 ^A | 94.6 ^A | 89.6 ^A | 81.6 ^B | 86.8 ^B | 84.5 ^B | 0.9 | < 0.001 | 0.13 | 0.55 |
| t9.25 | Aspartate | 89.2 ^y | 91.5 ^x | 88.2 ^y | 89.4 ^y | 93.0 ^x | 87.7 ^y | 77.7 ^y | 92.4 ^x | 81.2 ^y | 1.3 | 0.04* | 0.02 | 0.34 |
| t9.26 | Glutamate | 93.1 | 99.5 | 92.6 | 91.8 | 99.0 | 93.5 | 98.0 | 96.9 | 97.2 | 1.3 | 0.76 | 0.38 | 0.88 |
| t9.27 | Glycine | 89.9 ^a | 89.9 ^a | 94.8 ^a | 87.6 ^a | 92.8 ^a | 91.7 ^a | 68.3 ^b | 93.1 ^a | 88.1 ^a | 1.6 | < 0.001 | < 0.001 | < 0.001 |
| t9.28 | Serine | 82.4 ^{ab} | 87.9 ^a | 87.8 ^a | 89.1 ^a | 91.7 ^a | 84.9 ^{ab} | 67.3 ^b | 91.0 ^a | 81.2 ^{ab} | 1.7 | 0.02 | 0.01 | 0.04 |

Values are presented as mean ± SEM, $n = 3$. Values in the same row with different superscript letter differ significantly ($p < 0.05$): differences among treatments (a, b); for a particular alga, differences caused by technological process (x, y); and for a particular technological process, differences caused by algae (A, B). *without differences on post hoc test. **One-way ANOVA. NAN, *N. oceanica*; CHLO, *C. vulgaris*; TETR, *Tetraselmis* sp.; NO, not processed; PHY, physically processed; ENZ, enzymatically processed; NO, not processed; PHY, physically processed; ENZ, enzymatically processed. M, microalgae; P, process; EAA, essential amino acids; CEAA, conditionally essential amino acids; NEAA, nonessential amino acids; ND, not determined, when the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined

negative. Concerning individual EAA amino acids, it was observed that *N. oceanica* had the highest lysine ADC value, followed by *Tetraselmis* sp. and *C. vulgaris*. Threonine, phenylalanine, and aspartate's digestibility was significantly improved ($p < 0.05$) in all microalgae after physical processing. EAA ADC values were not significantly affected by the technological processing.

Discussion

The nutritional value of an ingredient for a certain fish species depends on its chemical composition but also on the bioavailability of its nutrients and energy, and this can be evaluated by their apparent digestibility coefficients, ADCs (NRC 2011).

To date, there is still few information concerning ADC values for most of the algae species that are emerging as possible ingredients for aquafeeds and this is a major step towards the formulation of nutritionally balanced diets for any fish species. The nutritional value of the algae used in this study was very variable among species, with *C. vulgaris* having the highest protein content (54%) followed by *N. oceanica* and *G. gracilis* (35%). *C. vulgaris* and *N. oceanica* had higher content of essential amino acids, EAA (> 20%), and were characterized by a high lipid content (10%), indicating that they could be good quality protein and lipid sources for aquafeeds. The inorganic matter (ash) was highest (> 30%) in *N. oceanica*, followed by *Tetraselmis* sp. and *U. rigida*, and these algae were particularly rich in Na, K, and Mg. The nutrient composition of both micro- and macroalgae has been

reported in literature and values vary greatly among species, cultivation strategies, seasons, and locations (Makkar et al. 2016; Neto et al. 2018; Tibbetts 2018), evidencing the need for an adequate nutritional evaluation of each lot prior use in aquafeeds. At the same time, the composition of farmed algae, as those used in this study, is rather consistent and their nutritional profile can be customized to meet the needs of the end product.

In this study physical-mechanical and enzymatic technological processes were applied to the no-processed algae to disrupt cell walls and promote the accessibility of intracellular nutrients. The physical-mechanical processing of the algae did not result in clear detectable changes in the protein bands profile of any of the five algae species as could be perceived by SDS PAGE, but when analyzed by size exclusion chromatography in the FPLC evidenced increased amount of low molecular compounds (peptide and amino acids) mainly in *U. rigida* and *C. vulgaris*. Moreover, the enzymatic processing clearly changed the protein profile of all algae, decreasing high-molecular-weight proteins and increasing the amount of low-molecular-weight ones. Both SDS PAGE and FPLC analysis evidenced that the enzymatic process was more effective than the physical-mechanical in changing the protein and peptides composition of the different algae, resulting in a particularly relevant increase of low-molecular-weight compounds especially in *U. rigida*, *G. gracilis*, and *N. oceanica*. Previous reports have shown that conventional mechanical and enzymatic methods for protein extraction may affect the integrity of extracted algal proteins due to the release of proteases from cytosolic vacuoles (Bleakley and Hayes 2017). Such intrinsic proteases could be partly responsible for the reduced presence of high-molecular-weight proteins after enzymatic processing as observed in the present study, especially for the *U. rigida*, *G. gracilis*, and *N. oceanica*, resulting in larger amount of low molecular compounds absorbing at 215 nm (peptide and amino acids) after enzymatic processing. These results are in general accordance with previous observations by Fleurence et al. (1995) reporting improved protein solubilization from edible seaweeds after the combined action of a polysaccharidase mixture (agarase and cellulase). Moreover, using a simulated in vitro gastrointestinal digestion model, Maehre et al. (2016) showed that enzymatic pre-treatment of seaweed biomass resulted in a 3-fold increase in amino acids available for intestinal absorption and could thus be an effective method for increasing the utilization potential of seaweed proteins. Nutrient accessibility was previously shown to play an important role in the nutrient digestibility in microalgae (Teuling et al. 2019), but this has to be confirmed by in vivo digestibility trials with target species.

In the present study, the formulation of the test diets followed a classic approach and was obtained by replacing 30% of a reference diet (REF) by the algal biomass, either before (entire, not processed algae, NO) or after physical-mechanical

(PHY) or enzymatic (ENZ) processing. The composition of each test diet largely reflected the composition of each algae resulting in a quite imbalanced composition (crude protein varying between 39 and 51% DM, crude fat between 9 and 14% DM, and gross energy between 19 and 25 kJ g⁻¹ DM). This is not an optimal approach but is the most widely used and accepted in nutritional trials (NRC 2011). According to our knowledge, very few studies evaluated the digestibility of either microalgae (Safari et al. 2016; Sarker et al. 2016; Tibbetts et al. 2017; Gong et al. 2018; Agboola et al. 2019; Teuling et al. 2019) or seaweeds (Pereira et al. 2012) in fish species, and in European seabass, studies are even scarcer (Valente et al. 2019a).

In our study, the dry matter, protein, and energy digestibility of the test diets containing *G. gracilis* did not differ from the REF diet, but the dietary inclusion of *U. rigida* negatively affected dry matter ADC values. *U. rigida*, when included at such high dietary inclusion level (30%), seems to have a lower nutritional value associated with its chemical composition and bioavailability of nutrients. The dry matter ADC reflects the digestible fraction of both organic and inorganic matter and is largely dependent on its insoluble carbohydrates and mineral composition. *Ulva rigida* has not only higher ash content but also a higher content of high-molecular-weight proteins compared to *G. gracilis* that may have contributed to the lower dry matter ADC value. Moreover, the complexity of algal polysaccharides in seaweeds may also have contributed to observed differences in digestibility and merits further evaluation. The lipid digestibility values presently reported for both seaweeds were highly variable and in some case might be considered an artifact probably due to the very low lipid content of the seaweeds (0.6–1.2%). There are no previous studies focused on the digestibility of seaweeds in European seabass, but Pereira et al. (2012) evaluated the ADCs of four different seaweeds, including *Ulva* spp. and *G. vermiculophylla* in rainbow trout (*Onchorynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). For both fish species, the dry matter ADC of the experimental diets was lower than that of the reference diet, but in rainbow trout, protein and energy digestibility were highest in *G. vermiculophylla*. Likewise, the present results showed that *G. gracilis* is better digested by European seabass than *U. rigida*. In fact, there was a significant effect of the tested seaweeds and technological process on nutrient digestibility. Dry matter ADC was increased by 19% in physically processed *G. gracilis* (GRA-PHY), contributing to a 22% increase in the energy ADC value. The EAA, CEAA, and NEAA digestibility values of GRA-PHY were the highest and contributed to the 4% increase in protein ADC. Although the increased ADC values of GRA-PHY were not significantly different from GRA-NO, we should keep in mind that this is a very short-term digestibility trial, so the dietary inclusion of this ingredient in a longer-term growth trial merits further consideration. Contrarily, both physical

and enzymatic processing technologies had a negative impact on *U. rigida* nutrient digestibility. Although the enzymatic process seems to be effective in increasing low-molecular-weight proteins, it might also have released complex polysaccharides that impaired nutrient digestibility. According to the literature, green algae cell wall is mainly constituted by polysaccharides (up to 54% of the algae dry weight) comprising both insoluble (cellulose, hemicelluloses, and lignin) and water-soluble sulphate polysaccharides, ulvan (8–29%). Ulvan seems to have an atypical gelling mechanism that may interfere with biological functions that are yet to be identified (Lahaye and Robic 2007). The negative value observed for dry matter digestibility, after *U. rigida* technological processing, suggests an antagonistic property of the test ingredient for the absorption of nutrients. This was particularly evident in some essential amino acids like leucine and methionine in which the digestibility was significantly reduced in processed *U. rigida*. In the case of methionine, a significant interaction was observed between the algae strain and the technological processing. But overall, results suggest that the tested processing methodologies do not seem to be appropriate to this alga species before its inclusion in diets for European seabass.

The dry matter digestibility of *N. oceanica* biomass in the test diets did not differ from the REF diet, but protein, lipid, and energy ADCs were significantly reduced in unprocessed algae (DNAN-NO). Likewise, in a digestibility study with Atlantic salmon, Gong et al. (2018) reported impaired protein (82 vs 86%) and energy (77 vs 83%), but not dry matter ADC values (67 vs 69%) in extruded diets with 30% defatted *Nannochloropsis* sp., compared to the reference diet. Untreated *Nannochloropsis gaditana*, also in a digestibility study, resulted in decreased dry matter, protein, lipid, and energy ADC values, in both African catfish (Agboola et al. 2019) and Nile tilapia (Teuling et al. 2019) compared to the reference diet. The only digestibility study performed in European seabass reported dry matter and protein ADCs of 68 and 85%, respectively, for defatted *Nannochloropsis* sp. (Valente et al. 2019a). These values are higher than those presently observed for no-processed *N. oceanica* but within the range of values observed for NAN-ENZ. The higher protein and lower fat content of defatted biomass, together with a possible positive effect of the defatting process on nutrient bioavailability may explain such differences. In the present study, and contrarily to seaweeds, a significant effect of the tested seaweeds, technological process, and interaction of both factors on nutrient digestibility was observed. Dry matter ADC more than doubled in both NAN-PHY and NAN-ENZ, but protein and energy ADCs have only increased with enzymatic processing (88 vs 82% and 87 vs 76%, respectively). These results suggest a higher effectiveness of enzymatic cell wall disruption to increase bioavailability of *N. oceanica* nutrients which is generally in accordance with the SDS PAGE

and FPLC data. The profiles of low molecular compounds from unprocessed and physically processed *N. oceanica* have high similarity, but when an enzymatic hydrolysis is applied to *N. oceanica*, the amount of low molecular compounds increased substantially. As an example, the amount of low molecular compounds, between 6.5 kDa (aprotinin) and 189 Da (Gly3), almost tripled. Moreover, increased soluble protein was reported in *Nannochloropsis* sp. after enzymatic hydrolysis (Valente et al. 2019b), which may partially explain the increased protein ADC presently observed. The digestibility of EAA was not significantly affected by the technological process (> 87%), but threonine and phenylalanine ADCs significantly increased in NANO-PHY. Curiously, the enzymatic process of *N. oceanica* has simultaneously increased the amount of peptides in the high-molecular end of the analysis, despite still being classified as low-molecular compounds (less than 20–30 kDa). We may hypothesize that this is a result of protein/peptide aggregation due to polysaccharide release, due to the release of proteins from the cell wall and/or cleavage of bigger (maybe insoluble) proteins into soluble peptides, and due to the action of cellulases. In any case, this might have increased nutrient accessibility and ultimately lead to increased protein and energy ADC values. The presence of intact cell wall seems a limiting factor for *Nannochloropsis* sp. digestibility in several fish species. Different cell wall disruption methods were used to increase bioavailability of *N. gaditana* nutrients for Nile tilapia, showing that bead milling the algae increased protein (78 vs 62%) ADC values in ingredient level, which were positively correlated with nutrient accessibility determined in vitro (Teuling et al. 2019). Moreover, in Atlantic salmon, extrusion processing significantly increased *Nannochloropsis* sp. dry matter ADC compared to cold-pelleting, but protein ADC remained unaffected (Gong et al. 2018).

The dry matter digestibility of *C. vulgaris* biomass in the test diets did not differ from the REF diet, but protein ADC was significantly reduced in unprocessed algae, evidencing the importance of cell wall disruption to improve nutrients digestibility. In fact, dry matter digestibility of *C. vulgaris* as single ingredient more than doubled in processed algae and protein ADC values increased 4% in CHLO-PHY (89 vs 86%) compared to unprocessed algae. The FPLC profiles did not reveal pronounced differences between processes applied to *C. vulgaris*, but an increase of low molecular compounds can be clearly observed in both technological processes and resulted in the highest protein ADC value for this species. Moreover, CHLO-PHY had generally high digestibility values for individual EAA, with threonine and phenylalanine ADCs having significantly higher ADC values than those observed for unprocessed algae (CHLO-NO). As far as we know, the digestibility of *C. vulgaris* has never been evaluated in European seabass, but in Atlantic salmon, previous studies demonstrated that dry matter, protein, lipid, and energy

digestibility dropped off in a relatively dose-dependent manner with the dietary inclusion of whole cell meal (Tibbetts et al. 2017). However, Tibbetts et al. (2017) have also shown that cell-rupture *C. vulgaris* biomass (by microfluidics), when included at 30%, could only significantly improve digestibility of dry matter and carbohydrates. This resulted in a protein ADC value of 85% for processed alga, which compares well with the present result for unprocessed *C. vulgaris* but is lower than values observed for either CHLO-PHY (89%) or CHLO-ENZ (88%). This difference may be explained by the comparatively higher protein and lower lipid content of the algal biomass used in our trial (54 vs 30% and 10 vs 26%, respectively). In fact, Tibbetts et al. (2017) predominantly related to the reduction of energy digestibility in Atlantic salmon fed 30% disrupted *C. vulgaris* to the dietary lipid fraction. But this could not be confirmed in our study as lipid and energy ADCs remained unaffected by the technological processing. In Nile tilapia, Sarker et al. (2016) reported a protein ADC of 80% for *Chlorella* sp. which is lower than the value presently reported for the unprocessed algae (86%) in spite of its equivalent biochemical composition. Authors attributed the low nutrient and energy ADC of *Chlorella* sp. to its high fiber content that might have inhibited proteolytic enzymatic activity. However, the present results evidenced the effectiveness of both the physical and the enzymatic processing of this microalga in improving protein and energy ADC, resulting in the highest values in CHLO-PHY and CHLO-ENZ.

Among tested algae, no-processed *Tetraselmis* sp. had the lowest protein (70%) and energy (49%) digestibility coefficients. The genus *Tetraselmis* is unique among the green algae in its cell wall formation; its cell body is covered by a solid cell wall (theca), formed by extracellular fusion of scales mainly composed of acidic polysaccharides (Arora 2016). In fact, SDS PAGE and FPLC results revealed limited differences in the amount of low molecular compounds between processed and unprocessed *Tetraselmis* sp., evidencing the strong resistance of these microalgae to disruption. However, the physical process of these microalgae was able to significantly improve protein and energy ADCs values by 20% and 66%, respectively. The digestibility of EAA was also significantly enhanced in processed *Tetraselmis* sp. (11–19% increase). This effect was particularly relevant in TETR-PHY that resulted in increased digestibility of threonine, isoleucine, leucine, valine, and phenylalanine with values above 85%. The negative lipid ADC values of *Tetraselmis* sp. stands out from the rest microalgae. This could either be an artifact resulting from the low lipid level of these algae, or could be associated to the high resistance of its cell wall structure to the digestive enzymes, which may inhibit lipid digestion. Tuelling et al. (2019) reported a significantly high correlation between fat ADC and hydrolysis degree ($r=0.94$), while Bitou et al. (1999) demonstrated that many marine algae inhibited the activity of pancreatic lipase. According to the literature, the

digestibility of *Tetraselmis* sp. has never been evaluated in fish as single ingredient, but a linear decline in nutrient digestibility was observed in European seabass fed diets with increasing levels of *Tetraselmis suecica* (Tulli et al. 2012). These results evidenced the difficulty of fish to access nutrients of this microalga, highlighting the need of technological processes prior its inclusion in aquafeeds. ADC values presently observed for several individual amino acids were significantly improved after physical technological processing of *Tetraselmis* sp., and in many cases with a significant interaction between tested seaweed and technological process. These results evidenced not only the efficiency of the alga processing in improving nutrient digestibility but also the need to select the most adequate method to disrupt the cell wall of each species.

In conclusion, the ability of European seabass to digest algae depends both on the selection of the most adequate algae species and on their technological processing. *Gracilaria gracilis* is better digested by seabass than *U. rigida*, and GRA-PHY merits further evaluation in long-term trials as resulted in the highest dry matter, protein, and energy ADCs. *Nannochloropsis oceanica* and *C. vulgaris* are better digested than *Tetraselmis* sp., and contrarily to seaweeds, their technological processing significantly affected nutrient digestibility. Protein and energy ADCs were highest in NAN-ENZ and CHLO-PHY, followed by TETR-PHY. Results clearly showed that it is possible to increase nutrient accessibility and digestibility of algae for European seabass, by selecting the most adequate method to disrupt the cell wall. It is also important to mention that, unlike many other experimental cell rupture methods reported in literature, the physical-mechanical and enzymatic technological processes used in this study are scalable to industrial level. Further studies are warranted to evaluate the potential of using such processed algae biomasses during long-term growth trials to fully address their potential as ingredients for aquafeeds.

Funding information Work supported by Project “MARINALGAE4AQUA - Improving bio-utilisation of marine algae as sustainable feed ingredients to increase efficiency and quality of aquaculture production”, financed by FCT, Foundation for Science and Technology, Portugal, through the research project ERA-NET COFASP/004/2015. Luisa Valente acknowledges national funds provided by FCT to CIIMAR (UIDB/04423/2020 and UIDP/04423/2020).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Agboola JO, Teuling E, Wierenga PA, Gruppen H, Schrama JW (2019) Cell wall disruption: an effective strategy to improve the nutritive

- quality of microalgae in African catfish (*Clarias gariepinus*). *Aquac Nutr* 25:783–797
- Araújo M, Rema P, Sousa-Pinto I, Cunha LM, Peixoto MJ, Pires MA, Seixas F, Brotas V, Beltrán C, Valente LMP (2016) Dietary inclusion of IMTA-cultivated *Gracilaria vermiculophylla* in rainbow trout (*Oncorhynchus mykiss*) diets: effects on growth, intestinal morphology, tissue pigmentation, and immunological response. *J Appl Phycol* 28:679–689
- Arora M (2016) *Tetraselmis*: an introduction. *Botanica* 66:155–175
- Atalah E, Cruz CMH, Izquierdo MS, Rosenlund G, Caballero MJ, Valencia A, Robaina L (2007) Two microalgae *Cryptocodinium cohnii* and *Phaeodactylum tricornutum* as alternative source of essential fatty acids in starter feeds for seabream (*Sparus aurata*). *Aquaculture* 270:178–185
- Batista S, Pereira R, Oliveira B, Baião LF, Jessen F, Tulli F, Messina M, Silva JL, Abreu H, Valente LMP (2020) Exploring the potential of seaweed *Gracilaria gracilis* and microalgae *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. *J Appl Phycol*. <https://doi.org/10.1007/s10811-10020-02118-z>
- Becker EW (2007) Micro-algae as a source of protein. *Biotechnol Adv* 25:207–210
- Bellou S, Baeshen MN, Elazzazy AM, Aggeli D, Sayegh F, Aggelis G (2014) Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnol Adv* 32:1476–1493
- Bitou N, Ninomiya M, Tsujita T, Okuda H (1999) Screening of lipase inhibitors from marine algae. *Lipids* 34:441–445
- Bleakley S, Hayes M (2017) Algal proteins: extraction, application, and challenges concerning production. *Foods* 6:33
- Bolin DW, King RP, Klosterman EW (1952) A simplified method for the determination of chromic oxide (Cr₂O₃) when used as an index substance. *Science* 116:634–635
- Campos I, Matos E, Aragão C, Pintado M, Valente LMP (2018) Apparent digestibility coefficients of processed agro-food by-products in European seabass (*Dicentrarchus labrax*) juveniles. *Aquac Nutr* 24:1274–1286
- Cho CY, Slinger S (1979) Apparent digestibility measurement in feed-stuffs for rainbow trout. In: Halver JE, Tiews K (eds) *Finfish nutrition and fish feed technology*, vol 2. Heenemann, Berlin, pp 239–247
- Demuez M, Mahdy A, Tomas-Pejo E, González-Fernández C, Ballesteros M (2015) Enzymatic cell disruption of microalgae biomass in biorefinery processes. *Biotechnol Bioeng* 112:1955–1966
- Fleurence J, Massiani L, Guyader O, Mabeau S (1995) Use of enzymatic cell wall degradation for improvement of protein extraction from *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata*. *J Appl Phycol* 7:393–397
- Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509
- Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl Å, Nelson R, Overturn K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac Res* 38:551–579
- Gong Y, Guterres HADS, Huntley M, Sørensen M, Kiron V (2018) Digestibility of the defatted microalgae *Nannochloropsis* sp. and *Desmodesmus* sp. when fed to Atlantic salmon, *Salmo salar*. *Aquac Nutr* 24:56–64
- Granby K, Amlund H, Valente LMP, Dias J, Adoff G, Sousa V, Marques A, Sloth JJ, Larsen BK (2020) Growth performance, bioavailability of toxic and essential elements and nutrients, and biofortification of iodine of rainbow trout (*Oncorhynchus mykiss*) fed blends with sugar kelp (*Saccharina latissima*). *Food Chem Toxicol* 141:111387
- Günkeren E, D'Hondt E, Eppink M, Garcia-Gonzalez L, Elst K, Wijffels R (2015) Cell disruption for microalgae biorefineries. *Biotechnol Adv* 33:243–260
- Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. *J Appl Phycol* 23:543–597
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Lahaye M, Robic A (2007) Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules* 8:1765–1774
- Lee A, Lewis D, Ashman P (2012) Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements. *Biomass Bioenergy* 46:89–101
- Maehre HK, Jensen IJ, Eilertsen KE (2016) Enzymatic pre-treatment increases the protein bioaccessibility and extractability in dulce (*Palmaria palmata*). *Mar Drugs* 14
- Makkar HPS, Tran G, Heuzé V, Giger-Reverdin S, Lessire M, Lebas F, Ankers P (2016) Seaweeds for livestock diets: a review. *Anim Feed Sci Technol* 212:1–17
- Maynard LA, Loosli JK, Hintz HF, Warner RG (1979) *Animal nutrition*. 7th edition edn., New York
- Monteiro M, Matos E, Ramos R, Campos I, Valente LMP (2018) A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass. *Aquaculture* 487:22–31
- Moutinho S, Linares F, Rodríguez JL, Sousa V, Valente LMP (2018) Inclusion of 10% seaweed meal in diets for juvenile and on-growing life stages of Senegalese sole (*Solea senegalensis*). *J Appl Phycol* 30:3589–3601
- Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Forster I, Gatlin DM, Goldberg RJ, Hua K, Nichols PD (2009) Feeding aquaculture in an era of finite resources. *Proc Natl Acad Sci U S A* 106:15103–15110
- Neto RT, Marçal C, Queirós AS, Abreu H, Silva AMS, Cardoso SM (2018) Screening of *Ulva rigida*, *Gracilaria* sp., *Fucus vesiculosus* and *Saccharina latissima* as functional ingredients. *Int J Mol Sci* 19 (10):2987
- NRC (2011) *Nutrient requirements of fish and shrimp*. National Academic Press, Washington, D.C.
- Ometto F, Quiroga G, Pšenička P, Whitton R, Jefferson B, Villa R (2014) Impacts of microalgae pre-treatments for improved anaerobic digestion: thermal treatment, thermal hydrolysis, ultrasound and enzymatic hydrolysis. *Water Res* 65:350–361
- Pereira R, Valente LMP, Sousa-Pinto I, Rema P (2012) Apparent nutrient digestibility of seaweeds by rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). *Algal Res* 1:77–82
- Pereira V, Marques A, Gaivão I, Rego A, Abreu H, Pereira R, Santos MA, Guilherme S, Pacheco M (2019) Marine macroalgae as a dietary source of genoprotection in gilthead seabream (*Sparus aurata*) against endogenous and exogenous challenges. *Comp Biochem Physiol C* 219:12–24
- Perez-Jimenez A, Abellan E, Arizcun M, Cardenete G, Morales AE, Hidalgo MC (2015) Nutritional and metabolic responses in common dentex (*Dentex dentex*) fed on different types and levels of carbohydrates. *Comp Biochem Physiol A* 184:56–64
- Rabilloud T, Charmont S (2000) Detection of proteins on two-dimensional electrophoresis gels. In: Rabilloud T (ed) *Proteome research: two-dimensional gel electrophoresis and identification methods*. Springer, Heidelberg, pp 107–126
- Robertson JB, Van Soest PJ (1981) The detergent system of analysis and its application to human foods. In: James WPT, Olof T (eds) *The analysis of dietary fiber in food*. Marcel Dekker Inc., New York, pp 123–158
- Sabeena Farvin KH, Baron CP, Nielsen NS, Otte J, Jacobsen C (2010) Antioxidant activity of yoghurt peptides: part 2 – characterisation of peptide fractions. *Food Chem* 123:1090–1097

- Safari O, Naserizadeh M, Mohammadi Arani M (2016) Digestibility of selected feedstuffs in subadult Caspian great sturgeon, *Huso huso* using settlement faecal collection and stripping methods. *Aquac Nutr* 22:293–303
- Sarker PK, Gamble MM, Kelson S, Kapuscinski AR (2016) Nile tilapia (*Oreochromis niloticus*) show high digestibility of lipid and fatty acids from marine *Schizochytrium* sp. and of protein and essential amino acids from freshwater *Spirulina* sp. feed ingredients. *Aquac Nutr* 22:109–119
- Silva DM, Valente LMP, Sousa-Pinto I, Pereira R, Pires MA, Seixas F, Rema P (2015) Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology. *J Appl Phycol* 27:1671–1680
- Skrede A, Mydland LT, Ahlstrøm Ø, Reitan KI, Gislerød HR, Øverland M (2011) Evaluation of microalgae as sources of digestible nutrients for monogastric animals. *J Anim Feed Sci* 20:131–142
- Teuling E, Wierenga PA, Agboola JO, Gruppen H, Schrama JW (2019) Cell wall disruption increases bioavailability of *Nannochloropsis gaditana* nutrients for juvenile Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 499:269–282
- Tibbetts SM (2018) The potential for ‘next-generation’, microalgae-based feed ingredients for salmonid aquaculture in context of the blue revolution. In: Jacob-Lopes E, Zepka LQ, Queiroz MI (eds) *Microalgal biotechnology*. IntechOpen, Riejea pp 151–175
- Tibbetts SM, Mann J, Dumas A (2017) Apparent digestibility of nutrients, energy, essential amino acids and fatty acids of juvenile Atlantic salmon (*Salmo salar* L.) diets containing whole-cell or cell-ruptured *Chlorella vulgaris* meals at five dietary inclusion levels. *Aquaculture* 481:25–39
- Tulli F, Chini Zittelli G, Giorgi G, Poli BM, Tibaldi E, Tredici MR (2012) Effect of the inclusion of dried *Tetraselmis suecica* on growth, feed utilization, and fillet composition of European sea bass juveniles fed organic diets. *J Aquat Food Prod T* 21 (3):188–197
- Tulli F, Pascon G, Niccolai A, Chini Zittelli G, Tredici M, Valente L (2017) Tibaldi E Effect of sonication on the nutrient digestibility of *Chlorella sorokiniana* in rainbow trout (*Oncorhynchus mykiss*). In: *Aquaculture Europe 2017*, Dubrovnik, Croatia
- Turchini GM, Ng W-K, Tocher DR (2010) Fish oil replacement and alternative lipid sources in aquaculture feeds. CRC Press, Boca Raton
- USEPA (1995). EPA method 3051: microwave assisted acid digestion of sediments, sludges, soils, and oils. Test methods for evaluating solid waste (third) USEPA, Washington, D.C
- Valente LMP, Araújo M, Batista S, Peixoto MJ, Sousa-Pinto I, Brotas V, Cunha LM, Rema P (2016) Carotenoid deposition, flesh quality and immunological response of Nile tilapia fed increasing levels of IMTA-cultivated *Ulva* spp. *J Appl Phycol* 28:691–701
- Valente LMP, Custódio M, Batista S, Fernandes H, Kiron V (2019a) Defatted microalgae (*Nannochloropsis* sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. *Fish Physiol Biochem* 45:1067–1081
- Valente LMP, Pintado MME, Batista SMG, Silva AMF, Faria JJA (2019b) Method for obtaining proteins or rich-protein extract from algae, extracts and uses therefore. Patent WO/2019/171293, PCT/IB2019/051811
- Van Soest PJ, Robertson JB (1985) Analysis of forages and fibrous foods a laboratory manual for animal science. Department of Animal Science, Ithaca
- Wan AHL, Davies SJ, Soler-Vila A, Fitzgerald R, Johnson MP (2019) Macroalgae as a sustainable aquafeed ingredient. *Rev Aquacult* 11: 458–492
- Wells ML, Potin P, Craigie JS, Raven JA, Merchant SS, Helliwell KE, Smith AG, Camire ME, Brawley SH (2017) Algae as nutritional and functional food sources: revisiting our understanding. *J Appl Phycol* 29:949–982
- Zheng L-X, Chen X-Q, Cheong K-L (2020) Current trends in marine algae polysaccharides: the digestive tract, microbial catabolism, and prebiotic potential. *Int J Biol Macromol* 151:344–354
- Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

- Q1. Please check if author names and affiliations are presented correctly.
- Q2. Please check if the section headings are assigned to appropriate levels.
- Q3. Kindly check if Tables are presented correctly.
- Q4. Ref. "Tuelling et al. (2019)" is cited in the body but its bibliographic information is missing.
Kindly provide its bibliographic information in the list.

UNCORRECTED PROOF